# Investigating the role of epidermal keratinocytes in the pathogenesis of systemic sclerosis

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by

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## Statement of Contribution

I, Joanna Nikitorowicz-Buniak, confirm that the work presented in the thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Joanna Nikitorowicz-Buniak

# Abstract

Systemic sclerosis (SSc) is an inflammatory connective tissue disorder of unknown aetiology, characterised by progressive fibrosis of skin and internal organs. The correct epithelial-mesenchymal interactions are essential for skin homeostasis and tissue repair, but little is known about these interactions in SSc. Moreover, epithelial cells were demonstrated to secrete cytokines and growth factors influencing phenotype and proliferation rate of fibroblasts. SSc epithelial cells have recently been shown to take on an activated phenotype similar to wound healing. Therefore, this thesis aims to further characterise SSc epidermis using immunohistochemistry, mRNA assays, and phosphoarrays. Furthermore evidence of SSc keratinocytes releasing factors capable of promoting fibrosis is explored, using immunoassays for proteins secretion and in vitro experiments on primary fibroblasts and HaCaT cells. Additionally, this thesis also addresses the question if SSc epidermis contributes to increased number of contractile fibroblasts via epithelial-to–mesenchymal transition.

This thesis demonstrates that the SSc epidermis has expanded thickness, and hypertrophied cells, as well as altered expression pattern of terminal differentiation markers. It also is releasing profibrotic and proinflammatory mediators, CTGF and S100A9, which are considered targets for novel therapies in rheumatology. In addition, it illustrates that S100A9 signalling via TLR4 is capable of promoting fibroblast responses in addition to its known proinflammatory effects. Also the CTGF induction and active profibrotic signaling is shown to affect cells in the upper dermis adjacent to the abnormally differentiated epidermis, consistent with cross-talk activation of the local fibroblasts. These changes in the epithelial layer may have a role in the SSc pathogenesis and contribute to the inflammation and fibrosis seen in the disease. Despite active Smad signaling seen to affect the adjacent papillary dermis in active SSc consistent with cross-talk, no evidence of full EMT in the SSc epidermis was found. However, I report changes consistent with partial EMT process resembling those seen during wound re-epithelialisation.

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# Publications Arising from this Thesis

## Peer Reviewed Journals

Abnormally Differentiating Keratinocytes in the Epidermis of Systemic Sclerosis Patients Show Enhanced Secretion of CCN2 and S100A9. J Nikitorowicz-Buniak, X Shiwen, D Abraham, C Denton, R Stratton. J Invest Dermatol. 2014 Jun 16. doi: 10.1038/jid.2014.253

## Manuscripts in preparation

Investigating The Role Of MRTF-A In Wound Healing And Systemic Fibrosis

Evidence of active TGFβ signaling with incomplete EMT in Systemic Sclerosis skin

## Presentations at International and National Conferences

Cytokines, Chemokines and Growth Factors Present in Conditioned Media from Epidermal and Dermal Explants of Healthy Controls and Systemic Sclerosis Patients. J Nikitorowicz-Buniak, S. W Xu, K Khan, D Abraham, C Denton, C Black, & R Stratton

- 11th International Scleroderma Workshop, 2010 (Poster presentation). *CLIN EXP RHEUMATOL, 28 (5)*, S72.
- British Society Rheumatology Annual Conference, 2011 (Poster presentation). *RHEUMATOLOGY, 50*, 130. OXFORD UNIV PRESS.

Analysis of Signal Transduction in Systemic Sclerosis Epidermis. J Nikitorowicz-Buniak, N Aden, C Denton, D Abraham, & R Stratton

- 12th International Scleroderma Workshop, 2011 (Poster presentation).
- British Society Rheumatology Annual Conference, 2011 (Poster presentation) *RHEUMATOLOGY, 50, 131. OXFORD UNIV PRESS*.

Absence of Epithelial to Mesenchymal Transition Despite Activation of Keratinocytes in Scleroderma Skin. J Nikitorowicz-Buniak, X. Shiwen, D Abraham, C Denton, C Black, & R Stratton

- American College Rheumatology Annual Conference, 2011 (Poster presentation). *ARTHRITIS AND RHEUMATISM, 63 (10), S581.*
- 3rd Systemic Sclerosis World Congress, 2012 (Poster presentation).
- British Society Rheumatology Annual Conference, 2012 (Poster presentation) *RHEUMATOLOGY, 51, 33*

Enhanced Release of S100A9 and Hepatocyte Growth Factor by The Epidermis in Systemic Sclerosis. J Nikitorowicz-Buniak, C Denton, D Abraham, & R Stratton,

- American College Rheumatology Annual Conference, 2012 (Poster presentation). *ARTHRITIS AND RHEUMATISM, 64 (10), S968*

Investigating The Role Of MRTF-A in Wound Healing and Systemic Sclerosis. R. Stratton, X. Shiwen, J. Nikitorowicz-Buniak, B. Abdi-Ahmed, M. Ponticos, C. Denton, D. Abraham, B. D. Smith.

- 13th International Scleroderma Workshop, 2013 (Poster Presentation).

Abnormally Differentiating Keratinocytes in Systemic Sclerosis Epidermis Show Enhanced Secretion of CCN2 and S100A9. J Nikitorowicz-Buniak, X Shiwen, C Denton, D Abraham & R Stratton

- The 7th International Workshop On The CCN Family Of Genes, 2013 (Oral Presentation)
- British Society Rheumatology Annual Conference, 2014 (Oral Presentation)
  - Young Investigators Award

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## List of Abbreviations

| | |
|---|---|
| αSMA | alpha smooth muscle actin |
| ACA | anti-centromere antibodies |
| ALK1 | activin receptor-like kinase type 1 |
| ATA | anti-topoisomerase antibodies |
| ARA | anti-RNA polymerase antibodies |
| BANK1 | B-cell scaffold protein with ankyrin repeats 1 |
| BMP | bone morphogenetic protein |
| BMPR2 | bone morphogenetic protein receptor type 2 |
| bp | base pair |
| COL1A2 | collagen type I, alpha 2 |
| CTGF | connective tissue growth factor |
| DAB | diaminobenzidine tetrachloride |
| DAMP | damage associated molecular pattern |
| dcSSc | diffuse cutaneous systemic sclerosis |
| DMEM | Dulbecco's modified Eagle's medium |
| EC | endothelial cell |
| ECM | extracellular matrix |
| ELISA | enzyme-linked immunosorbent assay |
| EMT | epithelial-mesenchymal transition |
| ET | endothelin |
| FCS | foetal calf serum |
| FFPE | formalin-fixed paraffin embedded |
| GVHD | graft versus host disease |
| GWAS | genone-wide association study |
| H&E | haematoxylin and eosin |
| HRP | horseradish peroxidase |
| IFN | interferon |
| IL-1 | interleukin-1 |
| IRF5 | interferon regulatory factor 5 |

| | |
|---|---|
| K | cytokeratin |
| kDa | kilo Dalton |
| lcSSc | limited cutaneous systemic sclerosis |
| LPS | lipopolysaccharide |
| MCP | monocyte chemoattractant protein |
| MHC | major histocompatibility complex |
| MMP | matrix metalloproteinase |
| mRSS | modified Rodnan skin score |
| NO | nitric oxide |
| NOS | nitric oxide synthase |
| OD | optical density |
| PAI-1 | plasminogen activator inhibitor-1 |
| PAMP | pathogen associated molecular pattern |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PDGF | platelet derived growth factor |
| P/S | penicillin and streptomycin |
| SD | standard deviation, |
| SEM | standard error of the mean |
| SMC | smooth muscle cell |
| SNP | single nucleotide polymorphism |
| SSc | systemic sclerosis, scleroderma |
| STAT4 | signal transducer and activator of transcription 4 |
| TβRI | type I TGFβ receptor |
| TG | transglutaminase |
| TGFβ | transforming growth factor β |
| TLR | toll-like receptor |
| Tsk1 | tight skin mouse |
| U3RNP | anti-fibrillarin |
| VEGF | vascular endothelial growth factor |

# Chapter One

# Introduction

## 1.1 Systemic sclerosis

### 1.1.1 Definition and classification

Systemic sclerosis (SSc) is a clinically heterogeneous multisystem rheumatic connective tissue disorder. A hallmark feature of SSc is, evolving over time, autoimmunity, inflammation, and vasculopathy, which lead to fibrosis (Kahaleh and LeRoy, 1999). Although, these processes are common to all forms of SSc, their extent varies between different forms.

As SSc clinical findings are often seen in other medical conditions The American College of Rheumatology (ACR) set specific criteria for the classification of SSc (LeRoy et al., 1988, 1980). They require meeting one major criterion or two minor criteria:

Major criterion:

• proximal diffuse sclerosis (skin thickening and tightening, non-pitting induration), which may affect the entire extremities, face, neck, and trunk.

Minor criteria:

• sclerodactyly fingers and/or toes
• digital pitting scars or loss of substance of the digital finger pads
• bibasilar pulmonary fibrosis.

However, recently the ACR/EULAR have modified the 1980 ACR criteria for SSc classification to ensure that more patients will be classified correctly as having the disease (van den Hoogen et al., 2013). It was decided that skin thickening of the fingers extending proximal to the metacarpophalangeal joints is sufficient for patient to be classified as having SSc. However, if that symptom is not present, other symptoms need to apply, such as skin thickening of the fingers, fingertip lesions, telangiectasia, abnormal nailfold capillaries, interstitial lung disease or pulmonary arterial hypertension, Raynaud's phenomenon, and SSc-related autoantibodies.

SSc patients are classified into subsets depending on the pattern of their skin involvement. The main forms are: diffuse cutaneous SSc (dcSSc) and limited cutaneous SSc (lcSSc). Other forms include overlap syndrome with features of other connective tissue disorders such as systemic lupus erythematosus (SLE), inflammatory arthritis, and systemic sclerosis sine scleroderma form with little or no skin sclerosis, despite other features of systemic sclerosis (Poormoghim et al., 2000). In addition there is a localized form, which involves one area of the skin and underlying tissues, most often developing in childhood (localized SSc, morphea).

#### 1.1.1.1 Limited cutaneous systemic sclerosis (lcSSc)

In lcSSc skin involvement is restricted to the extremities, but face and neck are usually also affected. Patients typically experience long history of Raynaud's phenomenon, often associated with recurrent digital ulcers. Other manifestations include cutaneous telangiectasis, subcutaneous calcinosis and oesophageal involvement. Lung fibrosis is less common, but vascular complications are seen more frequently. Patients are tested positive for anti-centromere antibodies however, anti-fibrillarin (U3RNP), anti-topisomerase-1 (ATA) and anti-RNA polymerase I and III antibodies may also be found.

#### 1.1.1.2 Diffuse cutaneous systemic sclerosis (dcSSc)

The classical early presentation of dcSSc is a sudden onset of inflammatory changes in the skin and internal organs. Pain and swelling of the extremities often occurs, and affected skin is typically itchy, with loss or change in hair growth. Symptoms of Raynaud's phenomenon develop simultaneously with other features or once the disease is established. Pulmonary, myocardial, and gastrointestinal tract involvement, as well as hypertensive renal crises, contribute to the significant morbidity and mortality. Whereas, the hallmark auto-antibodies for dcSSc include ATA, U3RNP and ARA. The skin sclerosis may remit after several years, despite progression of internal organ disease.

### 1.1.2 Epidemiology

SSc is a relatively rare disease, with estimated prevalence between 1 and 2 per 10,000 of population. Higher prevalence is recorded in North America and European ancestry population when compared to Asian origin groups (Barnes and Mayes, 2012). The incidence is estimated to be 1 in 100,000 per year (Mayes, 1997). As with most autoimmune diseases, female are 6 times more often affected than male (Silman et al., 1988). The only exception is environmentally triggered disease, which is more common in men (Silman and Jones, 1992).

### 1.1.3 Scleroderma pathogenesis

SSc pathogenesis is complex and not well understood. It is believed that an initial stimulus in a susceptible individual triggers a combination of immunological, inflammatory, and microvascular processes, which result in fibroblast activation and extracellular matrix (ECM) deposition, leading to skin and internal organ fibrosis (Jimenez and Bashey, 1977, Abraham and Varga, 2005, Abraham et al., 2007, Matucci-Cerinic et al., 2013).

Activated fibroblasts, in SSc lesions persist as myofibroblasts, with enhanced ability to produce collagens, adhere, and contract extracellular matrix (ECM) (Jimenez et al., 1986). Proposed mechanisms responsible for fibroblast activation include persistent and inappropriate expression of pro-fibrotic growth factors (Sato et al., 2000), stimulation by autoantibodies, cross talk from immune activated endothelial cells, and an important role for the epidermis (Aden et al., 2010). Mechanisms and feasibility of potential therapeutic targets were recently reviewed by Denton and Ong (Denton and Ong, 2013). Figure 1.1 summarises the interplay of inflammatory, fibrotic and vascular influences contributions to the disease.

It is unclear what initiates fibrotic process in the SSc skin. Factors suggested to be implicated in the pathogenesis of SSc include infection agents, environmental triggers

and genetic factors. Although link between infectious agents and SSc is not clear, several microbes were identified to play a potential role (Arnson et al., 2009). The most commonly associated include *Helicobacter pylori* (Radic et al., 2012, Yazawa et al., 1998), *Toxoplasma gondi* (Shapira et al., 2012), hepatitis B virus, cytomegalovirus (CMV) (Arnson et al., 2009), and parvovirus B19 (Ohtsuka and Yamazaki, 2004, Zakrzewska et al., 2009), as well as most recently Epstein-Barr virus (Farina et al., 2014).

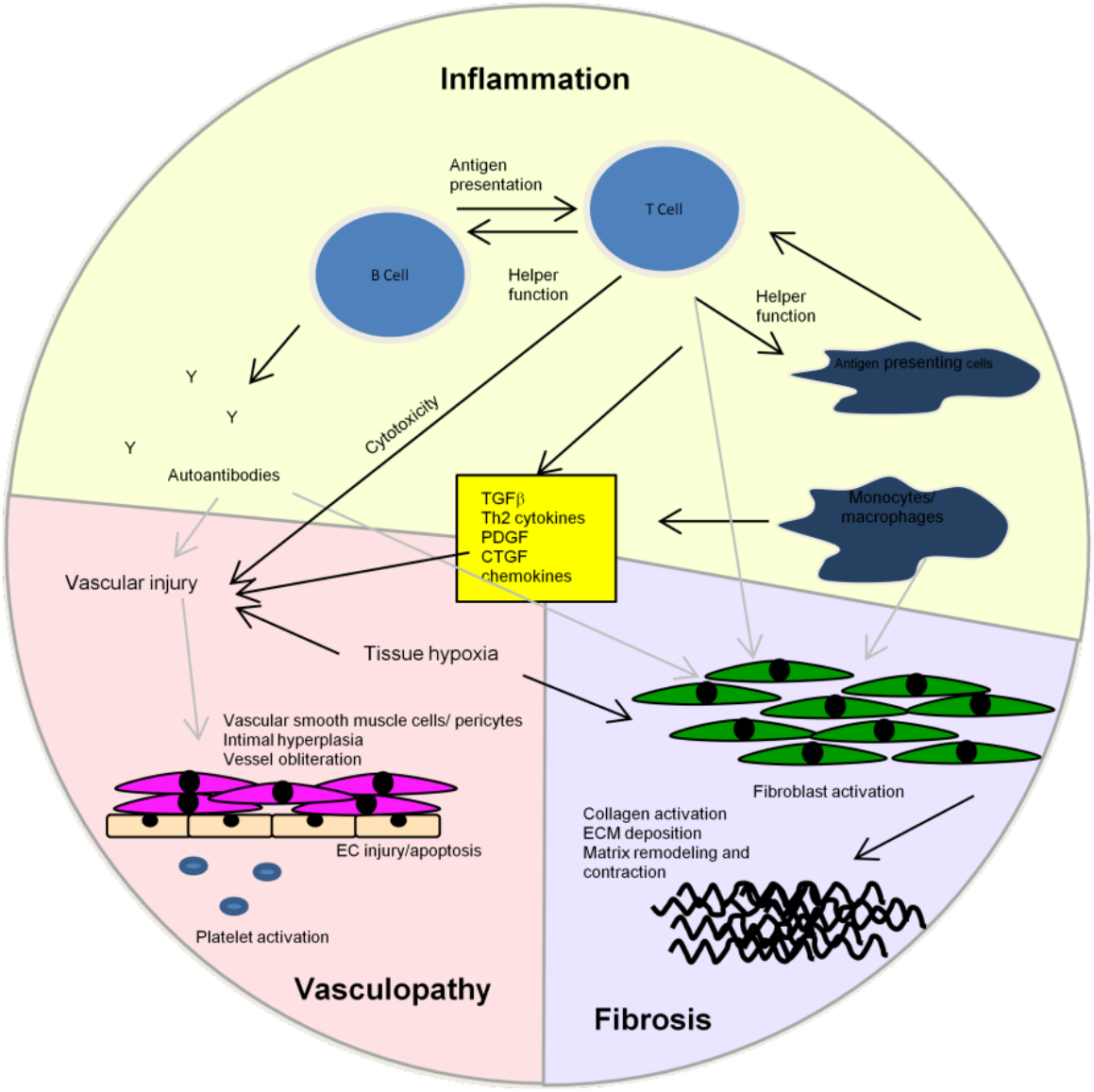


**Figure 1.1. Inflammatory, fibrotic and vascular contributions to the disease.**

from Derret-Smith 2012 (Derrett-Smith, 2013)

From environmental factors linked to occurrence of SSc exposure to polyvinyl chloride, silica dust, aromatic and aliphatic organic solvents, epoxy resins and formaldehyde are typically cited (Cowie, 1987, Silman and Jones, 1992, Nietert et al., 1999, Nietert and Silver, 2000, McCormic et al., 2010, Makol et al., 2011).

Some genetic predispositions for SSc have also been identified, however there are many conflicting reports. These differences might be due to ethnicity and discrepancies in particular subsets inclusion criteria. Most genes are linked to the inflammatory aspects of the disease with the strongest links to class II MHC haplotype (Gilchrist et al., 2001, Mayes et al., 2014). Other genetic associations with SSc include STAT4 (Dieude et al., 2009a, Rueda et al., 2009), IRF5 (Dieude et al., 2010, Dieude et al., 2009a, Dieude et al., 2009b), PTPN22 (Dieude et al., 2008, Diaz-Gallo et al., 2011), BANK1 (Dieude et al., 2009b, Rueda et al., 2010), CD247 (Radstake et al., 2010, Dieude et al., 2011), CTGF promoter (Kawaguchi et al., 2009, Fonseca et al., 2007), fibrillin-1 (Tan et al., 2001), IL-10 (Hudson et al., 2005), IL-1 (Kawaguchi et al., 2003), MCP1 (Carulli et al., 2008, Karrer et al., 2005) and COL1A2 (Hata et al., 2000). As well as more recently identified TNIP1 (Allanore et al., 2011), KIAA0319L, PXK and JAZF1 (Martin et al., 2013), and interferon- and transforming growth factor β-regulated genes (Christmann et al., 2014)

### 1.1.4 Skin involvement

Skin is an organ clinically affected in the vast majority of SSc cases and an early detectable marker of disease activity. The level of skin involvement in SSc patients is clinically assessed using the modified Rodnan skin score (mRSS) (Clements et al., 1993, Furst et al., 1998), which measure thickness of 17 areas at different body sites, grading them between 0 (normal) and 3 (severe thickening). The total skin score is a sum of individual skin assessment from all the sites and ranges between 0-51, the higher the score, the greater the extent and severity of skin thickening.

A link between the severity of skin involvement and visceral complications in dcSSc was first recognized in the 1960s and since then has been confirmed in a number of studies

(Clements et al., 2000, Shand et al., 2007, Steen and Medsger, 2001, DeMarco et al., 2002, Clements et al., 1990). Most recently the correlation between skin involvement and gene expression and the severity of interstitial lung disease was demonstrated (Assassi et al., 2013, Cottrell et al., 2014). The correlation between baseline skin score and disease severity and mortality in SSc patients, has been proposed as a useful disease predictor, and demonstrates the overall importance of skin pathology in the disease process (Clements et al., 2000). The majority of dcSSc patients develop the maximal skin score early in disease (Shand et al., 2007). Rapid increase in the mRSS in the early stage of disease is associated with a significantly increased risk of developing renal crisis (Steen et al., 1984). Consistent with skin involvement representing a window into this systemic disease, Steen and colleagues have demonstrated that, improvement in skin score of SSc patients is associated with improved survival (Steen and Medsger, 2001). Therefore, as a surrogate measure of disease severity, almost universally clinically affected, and an easily accessible organ, skin became important in studies on SSc.

Cutaneous manifestations usually start to develop on hands, and although they are mainly confined to the extremities they can extend to the trunk. Involved skin is characterised by oedema that is later replaced by thickened skin, followed by changes in skin pigmentation, alopecia (hair loss) and loss of sebaceous glands. Digital ulcers on the fingertips, contracted joints, restricted mouth opening and hardening of the mouth area (microstomia) are also among common SSc features (Denton and Black, 2004). Typical clinical presentations of dcSSc skin are shown in Figure 1.2.


**Figure 1.2. Typical clinical presentation of dcSSc.**

Classical features of dcSSc**: (a)** microstomia, **(b)** pigmentation changes, **(c)** skin tightening and **(d)** digital ulcers.

The histological changes, which occur in involved skin depend on disease duration and are similar to those observed in wound healing (Fig.1) (Aden et al., 2008). Early lesions contain inflammatory cell infiltrates and have up-regulated expression of pro-inflammatory cytokines such as IL-6 (Khan et al., 2012), IL-8, TNFα, CCL20 (Yamamoto, 2008), and MCP-1 (Tao et al., 2011), as well as increased levels of adhesion molecules, such as intracellular cell adhesion protein (ICAM) (Majewski et al., 1991), vascular cell adhesion protein (VCAM) or P-selectin (Koch et al., 1993). While in later stages the inflammation is resolved, and there is a loss of normal tissue architecture due to increased deposition of collagen and other extracellular matrix (ECM) proteins (Rodnan et al., 1979, Torres and Sanchez, 1998, Martin et al., 2012). In addition, blood vessels and sweat glands undergo atrophy, and subcutaneous fat layer may become reduced.

More recent report showed increased amount of collagen, myofibroblast accumulation, the mean epidermal thickness and the frequency of focal exocytosis, and the mononuclear cellular infiltration differed in clinically involved skin (Van Praet et al., 2011). Moreover, the authors demonstrated that all of the parameters except for mononuclear cellular infiltration, correlated with the skin score. In addition they have observed parakeratosis and proliferation of the intima in a minority of the SSc biopsies.

However, changes in SSc are not restricted to involved skin, studies of uninvolved skin from SS patients have also revealed differences from healthy skin. For instance reports have shown a significant decrease in fibrillin-1 (Wipff et al., 2010), and *MMP-1* while *TIMP-1*, and *HGF* genes expression increased (Frost et al., 2012) in clinically involved and uninvolved dcSSc skin in comparison to control skin. Furthermore, gene expression studies of SSc skin have demonstrated that patients may be sub-divided based upon their gene expression profiles: proliferating, inflammatory and fibrotic (Whitfield et al., 2003, Milano et al., 2008).

## 1.2 The epidermis

### 1.2.1 Definition

The epidermis is the outermost layer of the skin, the biggest organ in human body. It forms a mechanical and physical barrier protecting the body from water loss and pathogens. It consists of a stratified epithelium, constantly renewing under normal conditions and after injury, by maintaining a population of actively dividing cells in the hair follicles and innermost layer (Niemann and Watt, 2002). The cells in the epidermis are predominantly keratinocytes, but also include Langerhans cells, Merkel cells, and melanocytes.

Epidermal keratinocytes form four distinct layers: basal, spinous, granular, and cornified. As cells grow they migrate from basal location through the spinous, and granular layers, to the outermost cornified position, from which they are constantly shed. That change from proliferating keratinocyte into a dead, flattened part of stratum corneum is called cornification or terminal differentiation (Fuchs, 1990).

### 1.2.2 Terminal differentiation

Terminal differentiation begins when basal cells withdraw from the cell cycle and lose adherence to the basement membrane moving upwards towards the skin surface. In the spinous layers, cells strengthen their cytokeratin filaments framework, essential for mechanical strength, before moving into the granular layers (Candi et al., 2005). The outermost cornified layer consists of terminally differentiated, dead and flattened keratinocytes tightly attached to each other by corneodesmosomes, which become proteolytically degraded to facilitate desquamation (Simpson et al., 2011). Figure 1.3 shows a schematic of terminally differentiating epidermis including change in protein expression.

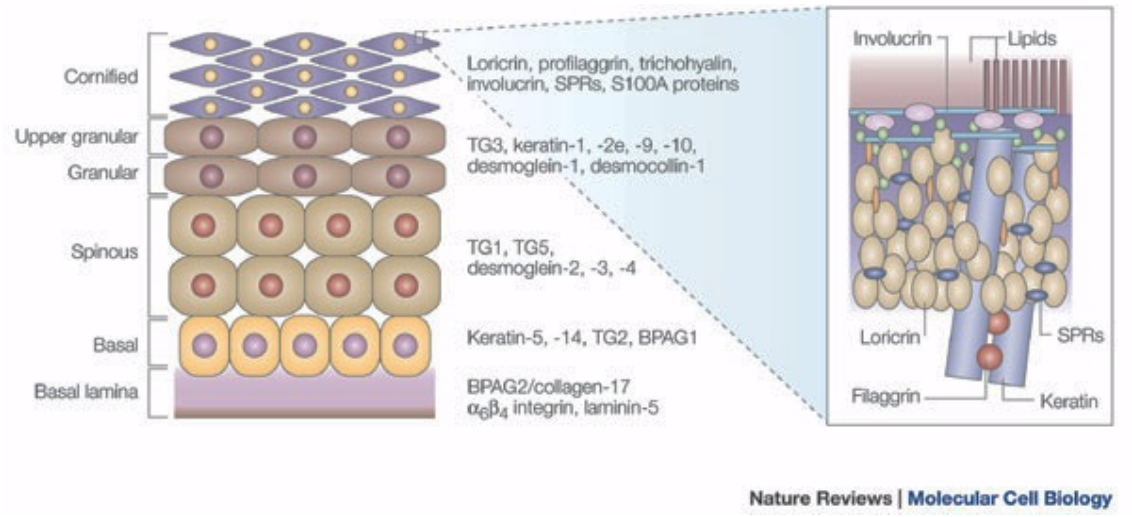


**Figure 1.3. Terminal differentiation of a healthy epidermis.**

TG-transglutaminase (from Candi et al., 2005)

Keratins K5 and K14 are the main structural proteins in proliferating keratinocytes that, assemble into keratin intermediate filaments. These filaments extend from desmosomes towards the nuclear lamina and together with tubulin and actin form cytoskeleton of the keratinocytes. Triggered by a signal, basal keratinocyte migrates towards the spinous layer losing its mitotic ability and beginning to synthesize new structural proteins and calcium dependent transglutaminase (TG) enzymes characteristic to cornification. The newly synthesized K1 and K10 replace K5 and K14. Later cells acquire keratohyalin granules containing pro-filaggrin, which during differentiation becomes proteolyticaly cleaved and released. Filaggrin aggregates keratin filaments into bundles leading to the collapse of the cells into a flattened shape characteristic to corneocytes in the stratum corneum. Simultaneously, cells produce other structural proteins such as loricrin and involucrin, which become cross-linked by calcium dependent TG1 and TG5. In addition, lipids, small proline-rich proteins and S100 proteins family members (S100A7, S100A10 and S100A11) are present in the assembled cornified envelope.

In normal epidermis the proliferation rate of basal keratinocytes layer is accurately balanced by desquamation of the cornified layer to constantly rejuvenate the epidermis. Several factors have been implicated as being able to switch the balance between proliferation and terminal differentiation with recent developments showing growing importance of ECM (Watt and Huck, 2013). Those factors promoting differentiation of keratinocytes, include increased Ca2+ concentration (Boyce and Ham, 1983, Celli et al., 2011), TGF-β (Matsumoto et al., 1990) and p38 MAPK signalling (Eckert et al., 2002), rounded cell shape (Watt et al., 1988), and decreased cell contact with the ECM (Watt et al., 1993) and physically restrained area for growth (Watt and Green, 1981). Whereas, HGF (Matsumoto et al., 1991) and JNK (Gazel et al., 2006) signalling was reported to inhibit differentiation of epithelial cells and promote keratinocytes proliferation.

Defects in the formation of the cornified envelope are responsible for disrupted epidermis homeostasis and result in disease, including lamellar ichthiosis in patients carrying mutation in *TG1* gene (Huber et al., 1995), Vohwinkel syndrome and progressive symmetric erythrokeratodermia linked to abnormal loricrin (Maestrini et al., 1996, Ishida-Yamamoto et al., 1997) and epidermolytic hyperkeratosis due to K1 and K10 mutations.

### 1.2.3 Epidermis in SSc and other fibrotic skin diseases

Recently the importance of epidermal genes on the phenotype was demonstrated in mouse mutants, showing not only implications in skin abnormalities but also indications of systemic effects (Liakath-Ali et al., 2014). There is increasing evidence supporting a key role for the epidermis in promoting dermal fibrosis in keloids (excessive scaring which extends beyond the initial site off injury), hypertrophic scaring (excessive scaring restricted to the initial site off injury) and SSc. Activated keratinocytes are found in hypertrophic scars as well as in SSc.

In hypertrophic scars decreased production of keratinocytes derived IL-1a (which stimulate matrix degradation) and increased PDGF (which increase matrix production)

was reported, which lead to decreased catabolism of dermal matrix and increased formation of ECM (Niessen et al., 2001). The result was replicated in another study, where co-culture of hypertrophic keratinocytes with normal wound myofibroblasts/ hypertrophic myofibroblasts/ fibroblasts induced thicker dermis due to increased synthesis of collagen and decreased MMP-1 as well as increased proliferation of dermal cells (Bellemare et al., 2005). Additionally, hypertrophic keratinocytes where shown to have increased secretion of TIMP-1, inhibitor of matrix degrading MMPs, which correlates with dermal thickness (Simon et al., 2011).

Whereas in keloids, keratinocytes where described to increase proliferation and decrease apoptosis of co-cultured fibroblasts (Funayama et al., 2003), as well as overexpress VEGF (Ong et al., 2007). They were also shown to have upregulated TGF-β receptors and Smad2/3 phosphorylation in co-cultured fibroblasts (Phan et al., 2005). Further studies demonstrated that fibroblasts co-cultured with keloid keratinocytes produced more ECM proteins and had increased levels of TGF-β activity (Xia et al., 2004). In other preliminary study our group showed that SSc epidermis promotes fibroblast activation and fibroblast CTGF expression via IL-1α, in 3-D co-culture (Aden et al., 2010). More recent findings show RXFP1 increased TLR4 expression in SSc keratinocytes, which was suggested to also play a role in activation of fibroblasts in SSc skin (Bhattacharyya et al., 2013). Whereas, cathepsin V, a proteolytic enzyme modulating angiogenic processes, collagen degradation and keratinocyte differentiation, were significantly decreased in SSc skin, including keratinocytes (Noda et al., 2013).

Although, the majority of studies over the last few decades were performed on the dermis, some studies showed changes in the epidermis. It remains unclear if the changes seen in the SSc epidermis are initiated by environmental insult or are secondary to dermal changes, such as altered properties of ECM.

Confocal laser scanning microscopy revealed that SSc epidermis is hypertrophic with a large number of melanocytes and increased melanin content (Sauermann et al., 2002). This confirmed previous observations of changes in skin pigmentation in SSc, which can

be severe and widespread (Tabata et al., 2000, Pope et al., 1996). TGF-β, which is important in epidermal-dermal interactions during wound healing and closely linked to SSc pathogenesis, was found to be expressed throughout the SSc epidermis (Rudnicka et al., 1994, Querfeld et al., 1999).

Immunohistological studies have also shown enhanced expression of cytokines (IL-6), adhesion molecules (VCAM and P-selectin in granular layer) and CD44 (in spinous and granular layers) in SSc epidermis (Koch et al., 1993). ET-1 (Tabata et al., 2000), monocyte chemo-attractant protein-1 (MCP-1) (Distler et al., 2001), VEGF (Davies et al., 2006) and IL-21 receptor (IL-21R) (Distler et al., 2005) have also been demonstrated to be up-regulated in the epidermis in SSc.

Previous data from our group have suggested that SSc epidermis is activated, with delayed keratinocyte differentiation and changes resembling those seen during wound healing (Aden et al., 2008). SSc keratinocytes express K6 and K16, which are normally present during wound healing but not in homeostasis. In addition K14 expression in SSc epidermis is not limited to the basal layer, but is expressed throughout epidermal layers (Figure 1.4), whereas K1/K10 expression is delayed. Also α6 integrin, which is responsible for the adhesion of basal cells to the basement membrane, is not restricted to basal cells in SSc.

Proteomic analysis demonstrated altered abundance of specific proteins in SSc skin, and pointed to increased expression of ECM proteins, proteins associated with contractility and mobility, but also oxidative stress and protein folding (Aden et al., 2008). Moreover, data showed activation of stress-induced mitogen-activated protein kinase (MAPK) in SSc and stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) in SSc epidermis as well as phosphorylation of c-Jun, and p38. Phosphorylation of HGF receptor, c-Met was also enhanced in involved skin of SSc patients (Aden et al., 2010).

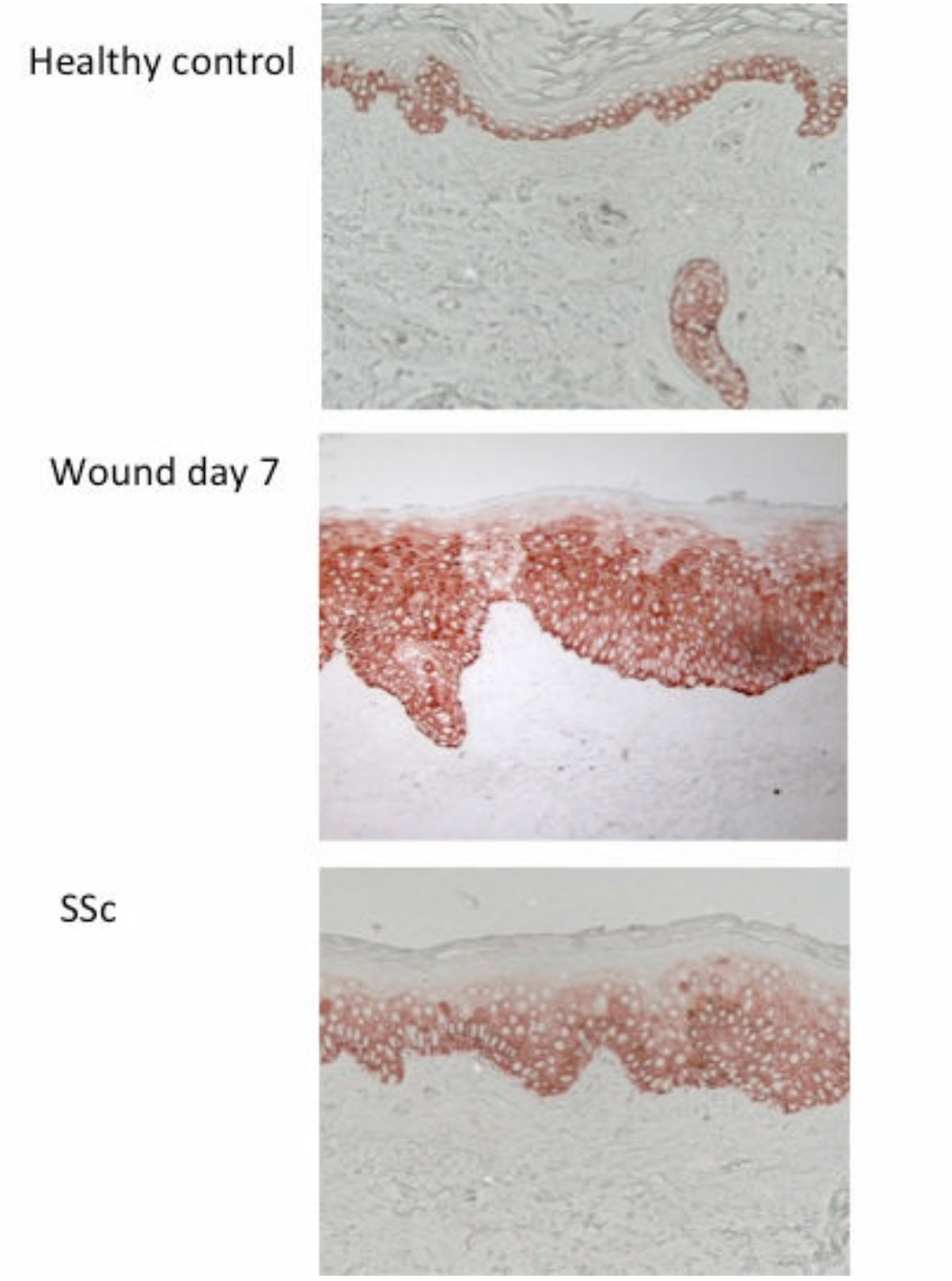


**Figure 1.4. Expression of CK14 in healthy and SSc skin and cutaneous wound.**
(from Aden et al., 2008)

## 1.3 Epithelial-mesenchymal interactions

### 1.3.1 Background

The epidermal-dermal cross-talk in healthy skin is well documented. Correct epithelial-mesenchymal interactions are essential in embryogenesis, skin homeostasis (Szabowski et al., 2000, Maas-Szabowski et al., 2000) and repair (Werner et al., 2007, El Ghalbzouri and Ponec, 2004). When disrupted it can result in pathogenesis, such as tumor formation (Bhowmick et al., 2004). These interactions are based on the production of soluble autocrine and paracrine factors, cell-matrix interactions (Gailit and Clark, 1994, Watt and Fujiwara, 2011), and direct cell-cell contact (Waelti et al., 1992).

Skin fibroblasts and keratinocytes are source of: growth factors - soluble molecules that influence growth and differentiation of cells, cytokines - signalling molecules released by cells in danger e.g. upon injury, and chemokines attracting immune cells of both adaptive and innate system to the site of injury/infection. They can act in autocrine, or paracrine manner or both. Their appropriate concentrations are essential for skin homeostasis and wound healing, and if the balance is shifted they create an environment for pathological events like fibrosis (Hogaboam et al., 1998). The key soluble factors involved in epithelial-mesenchymal cross-talk are summarised in a table below.

## Table 1.1. Key soluble factors involved in epithelial-mesenchymal cross-talk.

| Soluble factor | Cell type producing | Function | Relevance to fibrotic diseases |
|---|---|---|---|
| **Growth factors** | | | |
| **Fibroblast growth factor – 2 (FGF-2/ bFGF)** | Major source KC; others include FB, macrophages and EC | Angiogensis and FB proliferation during wound healing | ↑ in SSc serum (Hummers et al., 2009, Kadono et al., 1996) and SSc skin (Lawrence et al., 2006) ; ↑ in keloid skin (Funayama et al., 2003) |
| **Hepatocyte growth factor (HGF/SF)** | Upon injury by mesenchymal and EPC | Proliferation and migration of EPC during wound healing | ↑ in SSc serum (Beirne et al., 2009) and SSc skin (Frost et al., 2012); HGF ↓ collagen synthesis |
| **Vascular endothelial growth factor (VEGF)** | Upon injury mainly by KC and macrophages. | Angiogenic, mitogenic for EC; chemoattractant to monocytes, | ↑ in dcSSc skin KC (Davies et al., 2006) and skin of mouse scleroderma models (Zhou et al., 2007); ↑ in SSc serum (Hummers et al., 2009) |
| **Platelet derived growth factor (PDGF)** | Include EPC, EC and, FB at the site of injury | Proliferation, survival and migration of mesenchymal cells; ↑ collagen production and cell adhesion | PDGF-AA (Yamakage et al., 1992) and PDGF-BB and its receptor ↑ in SSc skin (Gay et al., 1989, Klareskog et al., 1990); ↑ in SSc serum (Hummers et al., 2009) |
| **Granulocyte macrophage-colony stimulating factor (GM-CSF)** | Include FB, EPC in response to immune activation and pro-inflammatory cytokines. | Stimulate KC and hematopoietic cells proliferation and differentiation | ↑ expression of GM-CSF receptor on SSc dermal FB (Postiglione et al., 2002); in SSc FB blocks deposition of type I collagen, and ↑ production of fibronectin and tenascin (Postiglione et al., 2005). |
| **Connective tissue growth factor (CTGF/CTGF)** | Include FB, EC and KC | Proliferation and migration of EC and FB, ECM production | Polymorphism associated with SSc (Fonseca et al., 2007, Kawaguchi et al., 2009, Kovalenko et al., 2009, Granel et al., 2010); ↑ in serum (Sato et al., 2000), plasma and dermal interstitial fluid (Dziadzio et al., 2005) and skin of SSc patients (Igarashi et al., 1996); constitutively overexpressed in SSc fibroblasts (Denton and Abraham, 2001). |
| **Cytokines** | | | |
| **Interleukin 1 alpha (IL-1α)** | Constitutively by EPC; upon stimulation by variety of cells including FB and immune cells | Proinflammatory; ↑ expression of antimicrobial peptides in KC; important in cross talk between KC and FB via AP-1 proteins. | Polymorphism in *IL1A* gene ↑ susceptibility to SSc and its severity (Kawaguchi et al., 2003); constitutively expressed by SSc FB (Kawaguchi, 1994); production associated with ↑ proliferation, pro-collagen synthesis, PDGF and IL-6 production (Kawaguchi et al., 1999); used by SSc KC to activate dermal FB (Aden et al., 2010); no significant difference in SSc patients sera when compared with healthy controls (Needleman et al., 1992). |

| | | | |
|---|---|---|---|
| **Interleukin 1 beta (IL-1β)** | | Proinflammatory; ↑ cell proliferation and differentiation | SSc FB show ↑ sensitivity to exogenous IL-1β (Kawaguchi et al., 1993); constitutively expressed by SSc FB (Kawaguchi, 1994) |
| **Interleukin 1 receptor antagonist (IL-1ra)** | Intracellular form (icIL-1ra) constitutively produced by KC during differentiation; extracellular form secreted by damaged KC and monocytes | Competitive inhibitor of IL-1, anti-inflammatory | IcIL-1ra ↑ in SSc FB (Higgins et al., 1999); might contribute to differentiation of FB into myofibroblasts and downregulation of collagenase expression. (Kanangat et al., 2006). |
| **Interleukin 6 (IL-6)** | Include KC, FB, EC and macrophages. | Important in early inflammation and wound healing. | ↑ in SSc FB (Kadono et al., 1998, Koch et al., 1993, Kondo et al., 2001) and serum of SSc patients (Gillitzer and Goebeler, 2001, Needleman et al., 1992); levels correlate with skin thickness score (Sato et al., 2001). |
| **Chemokines** | | | |
| **Interleukin 8 (IL-8)** | Several cell types | Proinflammatory, angiogenic, chemoattractant, activator of neutrophils, | ↑ expression by SSc FB (Kadono et al., 1998, Koch et al., 1993) and in serum of SSc patients (Codullo et al., 2011) |
| **Macrophage inflammatory protein 3 alpha (MIP-3α/ CCL20/ LARC)** | Constitutively at low levels by KC and EC; induced in other cell types including FB | Chemoattracts DC, T-cells and B-cells; important in skin and mucosa homeostasis and immune protection | ↑ along with its receptor CCR6 in early SSc skin (Tao et al., 2011). |
| **Monocyte chemoattractant protein -1 (MCP-1/CCL2/ MCAF)** | Monocytes and macrophages major source; other include EPC, EC and FB | Recruits monocytes, memory T-cells, NK cells eosinophiles, basophiles, DC and LC | Polymorphism in the promoter region linked with SSc (Karrer et al., 2005); ↑ in SSc patients serum (Hasegawa et al., 1999) and kidney fibrosis (Lloyd et al., 1997a, Lloyd et al., 1997b); ↑ collagen and TGFβ production by FB (Gharaee-Kermani et al., 1996) |
| **S100A9 (MRP14, calgranulin B)** | Neutrophils and monocytes; activated by environmental stress (Marionnet et al., 2003) and IL-1α (Bando et al., 2007) EPC and EC | Binds calcium, pro-inflammatory, antimicrobial, integrin synthesis and actin reorganization; ↓ KC proliferation and differentiation | ↑ in BAL of patients with idiopatic pulmonary fibrosis (Bargagli et al., 2011); profibrotic (Meyer et al., 2011, Shibata et al., 2004); ↑ in SSc patients saliva (Giusti et al., 2007); inhibits MMPs (Isaksen and Fagerhol, 2001), |

KC - keratinocytes; FB - fibroblasts; EPC - epithelial cells; EC – endothelial cells; DC - dendritic cells; LC - Langerhan's cells; BAL – bronchoalveolar lavage; ↑ - increase; ↓ - decrease;

In embryogenesis epithelial-mesenchymal interaction is critical; for example, TGF-β mediated cross-talk is involved in lung and palate formation (Shannon and Hyatt, 2004, Kaartinen et al., 1995). The importance of these interactions in adult skin started to emerge in 1970s, when Rheinwald and Green demonstrated that keratinocytes require the presence of fibroblasts for efficient growth (Rheinwald and Green, 1975). In the same year Rubin identified fibroblast-derived keratinocyte growth factor (KGF) and shown its ability to stimulate keratinocytes proliferation (Rubin et al., 1995). Later we learnt from epithelial-mesenchymal co-culture studies that keratinocytes can stimulate fibroblasts via interleukin 1 (IL-1) (Maas-Szabowski and Fusenig, 1996, Waelti et al., 1992) to secrete growth factors and cytokines essential for growth of epithelial cells, such as KGF, HGF (Weng et al., 1997, Gron et al., 2002), IL-6 or GM-CSF (Smola et al., 1993, Grossman et al., 1989). Critical for this paracrine loop is a transcription factor, activator protein-1 (AP-1) (Szabowski et al., 2000, Nowinski et al., 2004), regulating expression of genes responsible for keratinocytes proliferation and differentiation. Another growth factor released by keratinocytes is PDGF, which main role is to stimulate growth of fibroblast (Ansel et al., 1993).

Epithelial-mesenchymal interactions may also affect the phenotype of cells. For instance fibroblast feeders allow maintenance of stem cell populations within the basal cell layer of keratinocytes (Rheinwald and Green, 1975). Another example are fibroblasts, which become contractile much quicker in co-cultures with epithelial cells than in monolayers, due to the increased TGF-β release in such co-cultures (Shephard et al., 2004b). TGF-β, known to be released from keratinocytes after wounding, penetrates to the underlying dermis and stimulates fibroblasts to produce collagen (Yang et al., 2001). Another TGF-β superfamily member, activin also enhances ECM production and induces differentiation of fibroblasts into myofibroblasts; however, in contrast to TGF-β1 it does not seem to affect keratinocytes proliferation but induces terminal differentiation (Hubner et al., 1999). In contrary, IL-1α delays full differentiation of fibroblasts into contractile myofibroblasts (Shephard et al., 2004a, Shephard et al., 2004b).

The ultimate importance of epithelial-mesenchymal interactions was confirmed in microarray analysis of fibroblast gene expression after 48hr co-culture with keratinocytes. It showed up-regulation of 243 genes and down-regulation of 100 genes in response to keratinocyte-derived factors (Nowinski et al., 2004). The up-regulated genes included cytokines and chemokines (GRO-α, MCP-2, IL-8, G-CSF), proteins involved in proliferation (cyclin B1), pro-angiogenic (VEGF), regulating ECM production and remodeling (prolyl 4-hydroxylase, MMP-1 and -3, HAS-2, lysyl hydroxylase 2, fibromodulin and PAI-1). Whereas, tetranectin and WISP-2 were among genes shown to be down regulated. Unsurprisingly, most of the genes were regulated by keratinocytes derived IL-1α. Shepard's study on differential mRNA expression patter of fibroblast co-cultured with epidermal cells added to the up-regulated genes list, among others COX-22, MCP-1, α-SMA, calponin, ET-1, heparin binding EGF and CTGF (Shephard et al., 2004a).

Another example of close interactions between keratinocytes and fibroblast is synthesis of the basement membrane, separating epidermis from dermis, to which both types of cells contribute (Smola et al., 1998). Laminin 5 was shown to have epithelial origin while collagen VII, to be produced by both keratinocytes and fibroblasts (Marinkovich et al., 1993).

Epithelial-mesenchymal cross-talk is also based on cell-ECM interactions, which were recently reviewed by Watt and Eckes (Watt and Fujiwara, 2011, Eckes et al., 2010). The ECM provides scaffolding for the adhesion and migration of cells. ECM components include collagens, elastin, proteoglycans, fibronectin, laminins and proteins such as thrombospondin. Many of them can bind and modify activity of growth factors and cytokines, preventing them from degradation or act as reservoir from which they can be rapidly released (Flaumenhaft and Rifkin, 1991, Hynes, 2009). To contact with ECM cells use integrins and epidermal stem cells that are in the closest proximity to the dermis express the highest level of these receptors (Jones and Watt, 1993). Reduction of integrin expression or loss of contact with ECM triggers cell detachment from basal membrane and terminal differentiation (Watt, 2002). Loss of β1 and α3 integrins leads to impaired

wound healing and is associated with stronger inflammatory response, and enhanced TGF-β1 signalling (Grose et al., 2002).

Contact with ECM also transmits mechanical tension onto cells, which then modulates gene expression and leads to cell cytoskeleton reorganization. A combination of increased mechanical tension and TGF-β1 is responsible for differentiation of fibroblasts into myofibroblasts (Wipff et al., 2010) and enhanced collagen expression, forming an autocrine loop (Lindahl et al., 2002). Moreover, high tension down-regulates many proteases while up-regulating protease inhibitors (Chiquet et al., 2009). These observations clearly demonstrate that increased mechanical tension and up-regulation of ECM proteins, during wound healing and in fibrotic diseases, contributes to wound closure and scar formation and can further perpetuate pro-fibrotic changes.

### 1.3.2 Normal wound healing

If the skin barrier is breached, a wound healing process is initiated to re-establish its integrity. The wound healing cascade of events is highly regulated (Werner and Grose, 2003) and can be divided into three phases: blood clotting and inflammation, new tissue formation, and tissue remodeling (Gurtner et al., 2008). These phases although having a sequential order, overlap. The importance of wound healing and contribution of epithelial stem cells to the process was recently reviewed (Arwert et al., 2012)

Instantly after the injury until day 5 an inflammatory phase is induced, during which, blood and water loss are prevented by formation of a platelet plug followed by fibrin matrix. At the same time, inflammatory cells including neutrophils, monocytes and mast cells are attracted to the site of the injury. Inflammatory cells, protect from bacterial infection, remove dead tissue, but also secrete growth factors and cytokines. Low molecular weight compounds are also released from serum of injured blood vessels and degranulating platelets

On day 1-2 after injury, the second phase begins and lasts for up to three weeks. During this stage of wound healing, proliferation and migration of cells leads to formation of a new tissue. The process is initiated by absence of neighboring cells, release of mitogens (e.g. HGF, FGF-2 and EGF), and chemoattractants from degranulating platelets and infiltrating inflammatory cells, as well as up-regulation of their receptors. Keratinocytes from the wound edge and injured appendages become activated, release IL-1α and migrate forward between the epidermis and the fibrin clot, attaching to exposed collagen fibers. The leading edge keratinocytes change their integrin expression and release matrix metalloproteinase 1 (MMP-1) to allow degradation of the connective tissue on the way. Keratinocytes behind the leading edge proliferate and differentiate, restoring epidermal barrier function. Simultaneously, a latent form of TGF-β, bound to ECM becomes activated.

Shortly after, formation of new blood vessels (angiogenesis) is initiated, promoted by VEGF and FGF-2. Fibroblasts from the edge of the wound and attracted from the bone marrow proliferate, stimulated by PDGF and release factors stimulating re-epithelialisation, such as EGF, HGF and FGF. Soon after TGF-β induces fibroblasts differentiation into myofibroblasts, these then contract bringing the edges of the wound together. Along with fibroblasts, myofibroblasts synthesize ECM proteins, including fibronectin, and collagens type I and III. Simultaneously, new nerve endings develop around the wound edge. The resulting wound connective tissue is known as granulation tissue, because of the granular appearance of the numerous capillaries.

Finally, 2-3 weeks after injury the last remodeling phase begins and can last as long as 2 years. The activated cells return to a non-activated phenotype, some cells in the wound undergo apoptosis, others exit the wound, and a scar replaces the fibroblast-rich granulation tissue. Collagen remodeling during scar formation is dependent on continued synthesis and catabolism of collagen, controlled by MMPs secreted by epidermal cells, macrophages, endothelial cells, and fibroblasts themselves (Werner and Grose, 2003, Werner et al., 2007, Singer and Clark, 1999, Faler et al., 2006).

### 1.3.4 Fibroblasts and myofibroblasts

Fibroblasts are mesenchymal cells, which provide structural framework for many tissues and play a crucial role in the control of connective tissue homeostasis. When activated, they are the main cell type responsible for the synthesis of collagen and other ECM components, during wound healing and tissue fibrosis. Additionally, they contribute to tissue contraction and wound closure by generating fractional forces during migration into the wound and by differentiation into highly contractile myofibroblast (Tomasek et al., 2002, Desmouliere et al., 2005).

During fibrogenesis, fibroblasts become highly activated and undergo transition into myofibroblasts, with morphology and biochemical characteristics between those of the fibroblast and the smooth muscle cell (Gabbiani et al., 1971). Myofibroblasts express αSMA, as well as an array of stress fibers in their cytoplasm (Desmouliere et al., 2003), which allow them to remodel and contract ECM. Compared to fibroblasts myofibroblasts have also increased proliferation and migration, as well as enhanced ability to produce cytokines and ECM (Guarino et al., 2009, Desmouliere et al., 2003). Abundant quantities of myofibroblasts are present in sites of inflammation and tissue repair, where they contract connective tissue in order to close the wound (Guarino et al., 2009). However, the created scar is often stiff and disrupts function of the surrounding cells leading to tissue dysfunction and even organ failure (McAnulty, 2007). It has been suggested that it is a combination of TGFβ, IL-1β, and increased matrix stiffens that is responsible for a differentiation of fibroblasts into myofibroblasts (Leung et al., 2007).

### 1.3.5 Fibrosis as pathological wound healing

Physiological wound healing is self-limiting. A failure of the wound healing process to undergo normal resolution means that keratinocytes remain activated and myofibroblasts persist in the granulation tissue, continuing the deposition of ECM. These pathological events are thought to be a key to promote the development of fibrotic disorders such as

hypertrophic scarring, keloid formation and SSc (Abraham et al., 2007).

Normal fibroblast function, including their collagen production, is closely regulated in response to tissue requirement. Once sufficient ECM is laid down and re-epithelialisation is completed they either return to the quiescent state or undergo apoptosis. This ensures restoration of organ function, and allows cellular and collagen interactions, leading to gradual remodeling and strengthening of the scar (O'Kane and Ferguson, 1997). However, if fibroblasts remain activated, ECM secretion continues and tissue contraction persists, leading to matrix stiffening, formation of pathological scar and ultimately fibrosis (Kuhn and McDonald, 1991). Furthermore, fibroblasts from SSc lesional skin demonstrate capacity to produce more soluble collagen than the controls (LeRoy, 1974).

It is not well understood what causes the switch from normal responsive fibroblast to unresponsive, and persistently activated one, contributing to damaging fibrosis. Some of the possible explanations include persistent activation of fibroblasts by immune cells, environmental stress or cell injury, as well as inappropriate expression and/or persistence of fibrosis enhancing growth factors (Tomasek et al., 2002).

The origin of myofibroblast in fibrotic diseases such as SSc is disputable. Figure 1.5 shows proposed different ways of myofibroblast generation that include differentiated resident fibroblasts, epithelial and endothelial cells that underwent epithelial- and endothelial-to-mesenchymal transition, pericytes, or smooth muscle cells, as well as bone marrow derived circulating progenitor cells (fibrocytes) (Abraham et al., 2007, Hinz et al., 2007). However, recently, multiple lineage tracing was used to determine the origin of fibroblasts in kidney fibrosis lesions. The authors concluded that the majority of the myofibroblasts were derived from local fibroblasts, followed by recruitment of bone marrow derived cells, the endothelial-to-mesenchymal transition and the epithelial-to-mesenchymal (LeBleu et al., 2013).

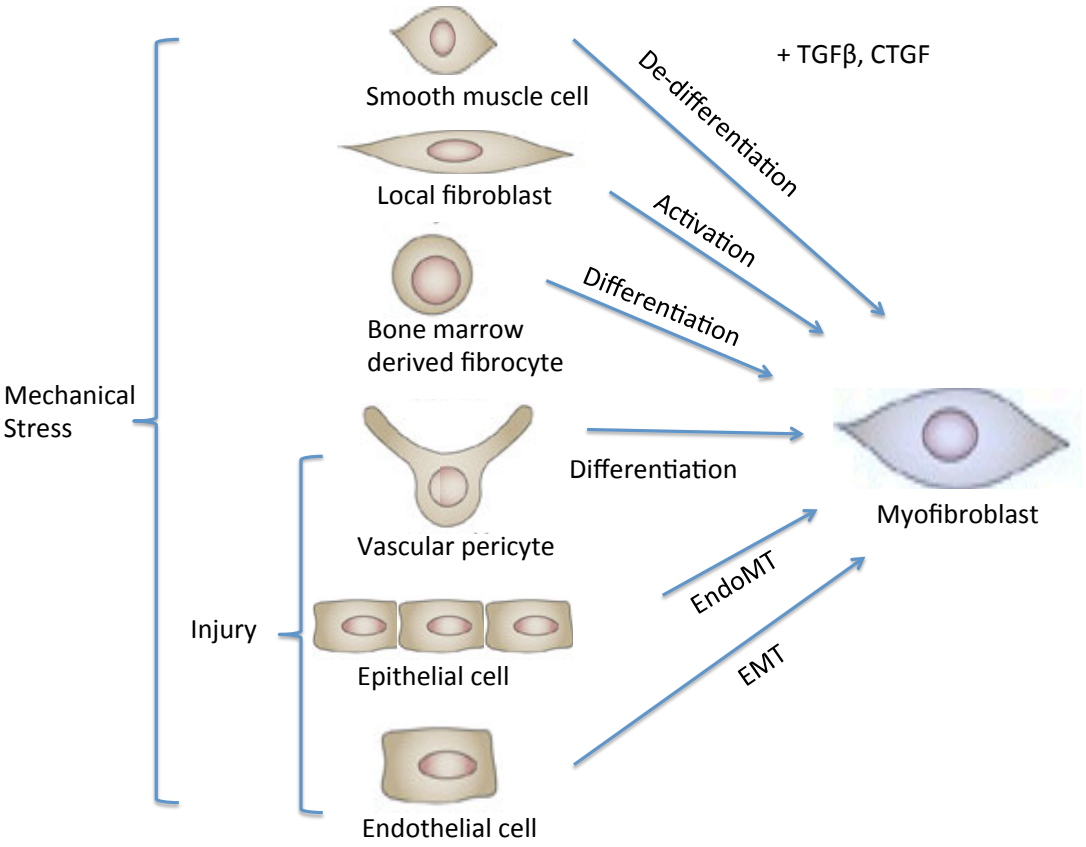


**Figure 1.5. Origin of myofibroblasts.**

## 1.4 Transforming Growth Factor β (TGF-β)

TGF-β is a multifunctional cytokine, belonging to a large superfamily, which apart from TGF-β proteins includes, bone morphogenetic proteins (BMPs), inhibins, activins, Growth Differentiation Factors (GDFs), Glial-derived Neurotrophic Factors (GDNFs), Nodal, Lefty, and Mülllerian Inhibiting Substance (MIS). A wide range of receptors is able to bind TGF-β superfamily ligands and transduce signal (Fig.1.6).

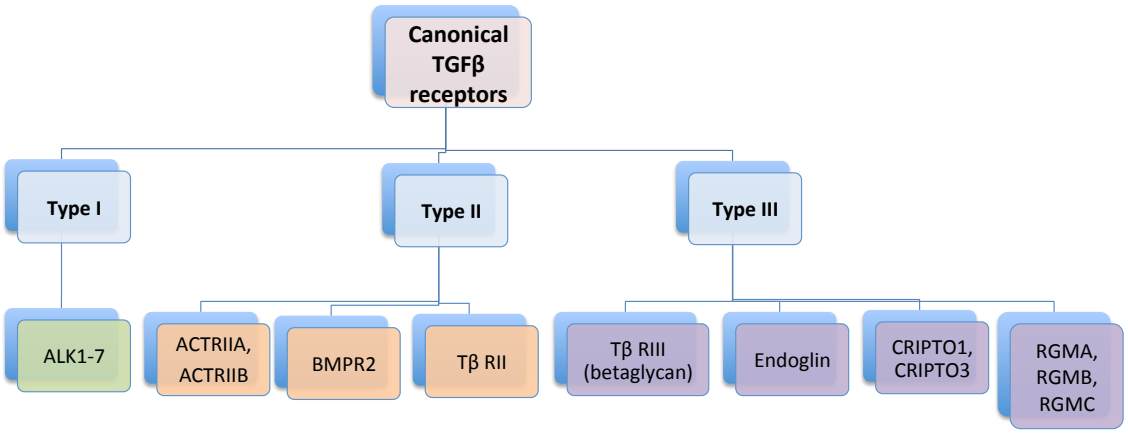


**Figure 1.6. Receptors transducing cannonical signalling of TGF-β superfamily.**

A family of mammalian TGF-β proteins consists of proteins designated TGF-β1–3. However, this thesis focuses on the TGF-β1 isoform most strongly linked to the development of fibrosis. The majority of cells including epithelial, stromal and immune cells can synthesize and have receptors for TGF-β. TGF-β is involved in many processes including wound healing, regulation of embryogenesis and tumors formation, immunity and inflammation, cell proliferation, differentiation and migration.

### 1.4.1 TGF-β signalling

The canonical TGF-β signalling pathway is Smad-dependent. In the absence of ligand, TGF-β receptor type 1 (TβRI) and TβRII are present as homodimers in the plasma membrane (Gilboa et al., 1998). After activation TGFβ binds TβRII leading to formation of a complex with TβRI. TβRII contains a constitutively active serine/threonine kinase, which phosphorylates and activates the kinase domain of the type I receptor, ALK5. ALK5 then phosphorylates cytoplasmic receptor associated Smads 2 and 3 (R-Smads), which form a heterodimer with Smad4, which is subsequently transported to the nucleus (Roberts, 1999). In the nucleus the Smad complex represses expression of some genes while activating others. Figure 1.7 shows a schematic representation TGF-β signalling pathway.

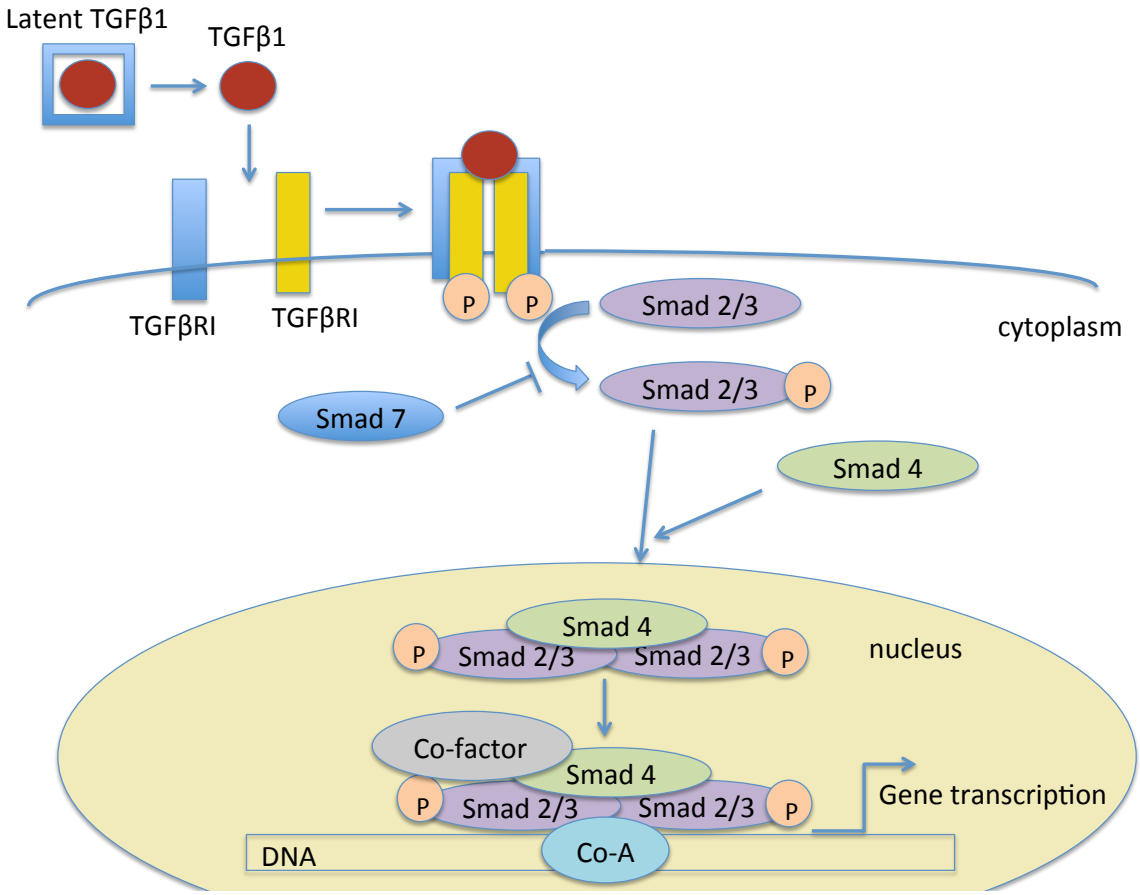


**Figure 1.7. Cannonical TGF-β signalling pathway**

The high degree of signalling specificity and versatility of TGF-β is possible due to availability of different combinations of interactions in a cell and context dependent fashion. The signalling responses can be regulated by differential type I and type II receptor interactions, Smad complex formation, receptor and Smad interactions with accessory proteins, and crosstalk of the Smads with other signalling pathways.

However, TGF-β can also signal via non-canonical pathways such as MAPK. Abnormal TGF-β signalling is associated with several fibrotic diseases, including SSc, where constitutive activation of R- Smads in fibroblasts was reported (Ihn et al., 2006).

### 1.4.2 Regulation of the TGF-β

Due to powerful pleiotropic effects of TGF-β, its signalling must be closely regulated. There are mechanisms contributing to both enhancement and suppression of these responses. The principal way, in which biological activity of TGF-β is controlled, is its release in latent form. The latent precursors, called latent TGFβ complex (LTGF-β), consists of a mature TGF-β dimer non-covalently bound to its latency associated peptide (LAP). In addition LTGF-β can also be coupled with latent TGF-β binding proteins (LTBP) (Rifkin, 2005).

TGF-β becomes activated after proteolytic cleavage of LAP and LTBP (Javelaud and Mauviel, 2004) by several proteases such as, thrombospondin-1 (Schultz-Cherry and Murphy-Ullrich, 1993), MMP-2 and-9 (Sato and Rifkin, 1989, Yu and Stamenkovic, 2000), or by binding to integrin $\alpha_v\beta_6$ (Annes et al., 2003). Moreover, the exposure to reactive oxygen species was also shown to regulate TGF-β signalling (Jain et al., 2013).

Downstream TGF-β signalling leads also to expression of proteins enhancing cellular responses to TGF-β, and therefore further increasing wound healing and fibrosis. They include ED-A fibronectin and CTGF, known to promote fibroblast proliferation, matrix production, granulation tissue formation, migration, adhesion and contraction of collagen matrix (Shi-wen et al., 2000, Chen et al., 2001, Crean et al., 2002). In contrary, tumor necrosis factor-α (TNF-α) and interferon-ϒ (IFN-ϒ) suppress TGF-β signalling. TNF-α was shown to repress Smad3-dependent signalling, as well as TGF-β induction of collagen and CTGF (Abraham et al., 2000). IFN-ϒ inhibits TGF-β induced collagen synthesis (Higashi et al., 2003).

TGF-β is rapidly induced after injury and can be detected throughout the wound healing process (Kane et al., 1991). Its release enhances attraction of immune cells and fibroblasts to the site, which then release more TGF-β. TGF-β also inhibits proliferation of epithelial cells, but stimulate fibroblast proliferation and ECM synthesis (Trojanowska, 2002), simultaneously inhibiting ECM degradation by down-regulating MMP-1, and up-

regulating tissue inhibitors of MMPs (TIMPs) (Ihn, 2005). Although beneficial in wound healing, in other situations excess collagen deposition by fibroblasts leads to scar formation and fibrosis which is a feature of many connective tissue diseases, including SSc (Krieg et al., 2007).

### 1.4.3 TGF-β in SSc and other fibrotic diseases

The accumulation of collagen in tissue fibrosis is a consequence of an imbalance between enhanced production and deposition, and impaired degradation of ECM. TGF-β is one of the key drivers of fibrosis, responsible for creating such an imbalance. It facilitates differentiation of fibroblasts into ECM overproducing myofibroblasts, while it simultaneously decreases levels of ECM degrading MMPs (Varga and Jimenez, 1986, Overall et al., 1989). Moreover, it also decreases proliferation of epithelial cells (Murphy et al., 1991), which is known to lead to impaired wound healing and contributes to scaring tissue formation.

Increased levels of TGF-β and its receptors were reported in fibroblasts form hypertrophic scars (Schmid et al., 1998), keloids (Chin et al., 2001), as well as at the leading edge of dermal lesions of SSc patients (Querfeld et al., 1999). Moreover, increased phosphorylation of Smad3 in keloids and SSc strongly suggests the importance of TGF-β/Smad signalling in pathogenesis of these fibrotic skin diseases (Phan et al., 2005, Verrecchia et al., 2006, Mori et al., 2003). However, results of the studies on the early and established SSc have demonstrated, Smad mediated TGF-β involvement in the initiation of fibrotic change, but Smad-independent maintenance of disease (Holmes et al., 2001).

Additionally, a reduced expression of inhibitory Smad7 was reported in involved skin of SSc patients when compared to uninvolved skin of the same patient (Dong et al., 2002). Still, other studies have shown no difference in the expression of Smad7 by dermal fibroblasts (Holmes et al., 2001, Mori et al., 2003). Animal studies have shown that

Smad3 deletion protects animals form cutaneous fibrosis (Flanders et al., 2002), while levels of nuclear Smad3 are elevated in animal models of fibrosis (Takagawa et al., 2003). Moreover, TGF-β also stimulates epithelial cells to undergo epithelial to mesenchymal transition (EMT) (Zavadil and Bottinger, 2005), which was also shown to contribute to fibrosis (Guarino et al., 2009, Takahashi et al., 2013).

### 1.4.4 Epithelial-mesenchymal transition (EMT)

#### 1.4.4.1 Definition

EMT is an important mechanism of embryo- and organogenesis(Thiery and Sleeman, 2006), and in adult life is reactivated during inflammation and wound healing. However, studies also showed involvement of EMT in pathological processes, such as fibrosis and formation of many types of cancer and their metastasis (Kalluri and Neilson, 2003, Kalluri and Weinberg, 2009, Lopez-Novoa and Nieto, 2009). Both developmental and pathological EMTs are characterised by a spectrum of changes in morphology, gene expression and signalling pathways.

During EMT epithelial cells undergo changes including, loss of cell-to-cell contact, due to decreased expression of proteins responsible for attachment, such as E-cadherin or desmoplakin; up-regulation of matrix degrading proteases, which digest the epithelial basement membrane allowing cell migration through the basement membrane; actin re-arrangement leading to change in cell shape to spindle and fibroblast like. As an effect of these changes, epithelial cells lose their epithelial characteristics and acquire mesenchymal cells properties. These changes seem to be Smad-3 dependent and are correlated with decreased expression of epithelial cell markers and induction of mesenchymal cell markers and transcription factor Snail (Hoot et al., 2008). There are several examples where factors implicated in EMT are known to be abnormally expressed in SSc and they include:

- Growth factors and cytokines: TGF-β, EGF, HGF, FGF and CTGF

- ECM components through integrins
- Wnt proteins, Notch
- Hypoxia
- ROS
- Mechanical stress

#### 1.4.4.2 Main characteristics of epithelial and mesenchymal cells

In typical epithelium, cells form a sheet, within which they are closely connected by: intracellular junctions, such as tight junctions, desmosomes, adherent junctions and gap junctions. This allows the epithelial sheet to form three-dimensional (3D) structures and keep the structural integrity, but also inhibit cells movement and dissociation from the epithelium. Another characteristic of epithelial sheets is polarisation in an apical-basal pattern, which results in differences between cells, depending on their location within the epithelium. In contrast, mesenchymal cells do not form regular layers of cells or form intracellular junctions. Mesenchymal cells have elongated shape, end-to-end polarity and can migrate freely within tissues and produce ECM.

#### 1.4.4.3 Types of EMT

EMT can be divided into 3 major types, type 1 seen in embryogenesis and gastrulation, type 2 observed in wound healing and fibrosis, and type 3 leading to cancer formation (Kalluri and Neilson, 2003, Zeisberg and Neilson, 2009). Each type is characterised by different events and expression of proteins. Presence of certain EMT markers is common to all types, however others are expressed only in particular types of EMT (Zeisberg and Neilson, 2009). A short characteristic of the main EMT types and their markers are presented in the Table 1.2 (adapted from Zeisberg et al., 2009).

**Table 1.2 Types and markers of EMT.**

| | Type 1 EMT | Type 2 EMT | Type 3 EMT |
|---|---|---|---|
| **Characterisation** | Epithelial-mesenchymal transitions; primitive epithelial cells transform into mesenchymal cells | Epithelial – fibroblast transitions; secondary epithelial/ endothelial cells transform into fibroblasts inflammation | Carcinoma-metastatic transitions; epithelial carcinoma cells migrate and form secondary tumor nodule |
| **Examples** | Embryogenesis, gastrulation | Wound healing (transient and self limiting EMT) and fibrosis (persistent EMT) | Tumor metastasis |
| **Markers of EMT** | | | |
| Cell surface | | | |
| N-cadherin | Acquired | Acquired | NA |
| E-cadherin | Diminished | Diminished | Diminished |
| ZO-1 | Diminished | Diminished | Diminished |
| Cytoskeletal | | | |
| FSP-1 | Acquired | Acquired | Acquired |
| α-SMA | NA | Acquired | Acquired |
| Vimentin | Acquired | Acquired | NA |
| Cytokeratin | Diminished | Diminished | Diminished |
| ECM proteins | | | |
| Type I collagen | Acquired | NA | Acquired |
| Type III collagen | Acquired | NA | Acquired |
| Type IV collagen | Diminished | Diminished | Diminished |
| Fibronectin | Acquired | Acquired | NA |
| Laminin 1 | Diminished | Diminished | Diminished |
| Laminin 5 | Acquired | Acquired | NA |
| Transcription factors | | | |
| Snail 1 | Acquired | Acquired | Acquired |
| Snail 2 (Slug) | Acquired | Acquired | Acquired |
| ZEB1 | Acquired | Acquired | Acquired |
| FOXC2 | Acquired | Acquired | NA |
| Ets-1 | Acquired | Acquired | Acquired |
| Twist | Acquired | Acquired | Acquired |
| Goosecoid | Acquired | Acquired | NA |
| MicroRNAs | | | |
| miR200 | Diminished | Diminished | Diminished |
| miR205 | Diminished | Diminished | Diminished |
| miR155 | Increased | NA | Increased |
| miR21 | Increased | Increased | Increased |
| miR29 | Increased | Increased | Increased |
| miR9 | Increased | Increased | Increased |

To demonstrate EMT process a few criteria should be met simultaneously. They include switch from E-cadherin to N-cadherin, at least partial loss of epithelial markers (e.g. cytokeratin) with gain of mesenchymal markers (e.g. vimentin, FSP-1), as well as phenotypic changes characteristic to EMT: shift to spindle shape morphology and loss of polarity, enhanced migration and resistance to apoptosis. In addition, cell should maintain the phenotype after removal of the stimuli.

However, apart from the 3 main types of EMT mentioned above a partial EMT was also identified. During a complete EMT, epithelial cells undergo an extreme change in their phenotype to become mesenchymal cells. While, during a partial EMT, epithelial cells do not undergo the whole transition process into a fibroblasts, and are characterized by an intermediate phenotype. A phenotype consistent with partial EMT has been described in wound healing, where cell-cell contact become loose, and cells can still revert to epithelial phenotype (Arnoux et al., 2008), and breast cancer where epithelial cells express several of EMT markers, while still maintaining epithelial phenotype (Sarrio et al., 2008, Chao et al., 2012), as well as in renal and liver fibrosis (Iwano et al., 2002, Zeisberg et al., 2007).

#### 1.4.4.4 Relevance of EMT in fibrosis

A persistent inflammation leads to release of cytokines into the microenvironment of epithelial cells, leading to their activation. Furthermore, it can cause epithelial cells to lose polarity and undergo transition into fibroblasts. Cells can then migrate through the degraded basement membrane. Originated in this way fibroblasts become activated by inflammatory signals and start to produce ECM, which accumulate leading to tissue fibrosis (Fig 1.8).

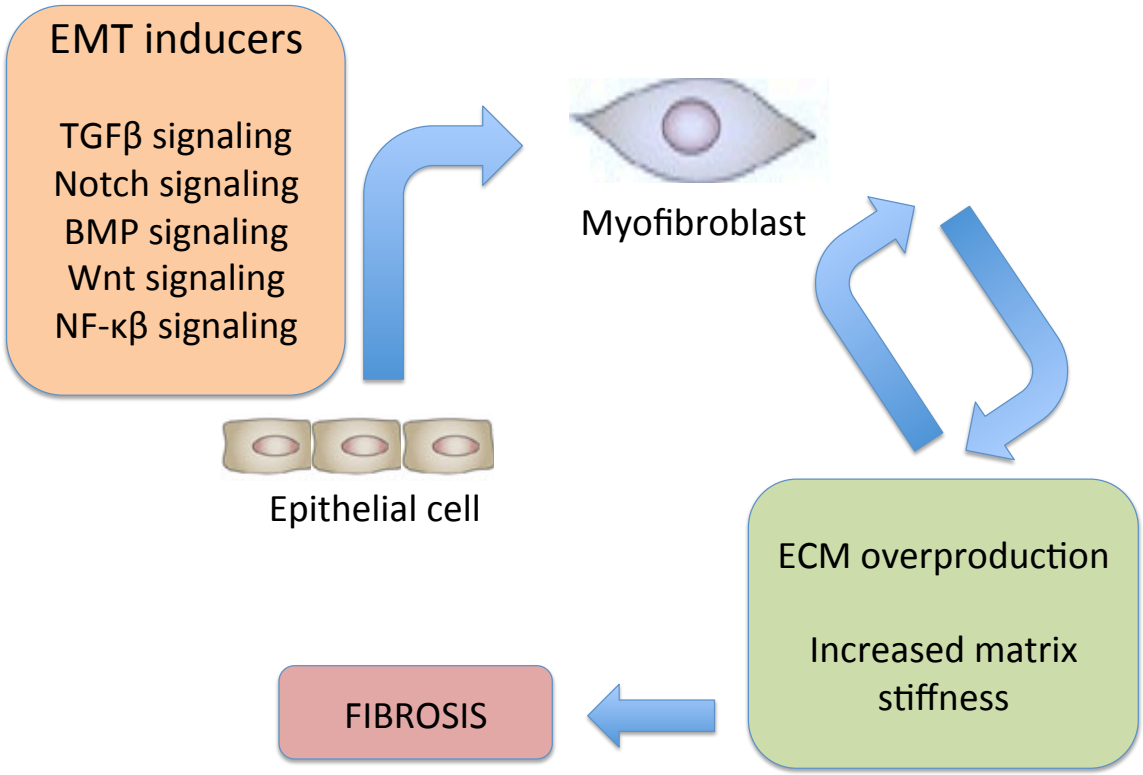


**Figure 1.8. EMT in fibrosis.**

Implication of EMT process in fibrosis of different organs was discovered (Guarino et al., 2009, Flier et al., 2010), including skin (Takahashi et al., 2013). A link between EMT and lung fibrosis is well established (Kim et al., 2006, Chilosi et al., 2003, Willis et al., 2005, Yasukawa et al., 2013), but in case of fibrosis of other organs, such as kidney it is more disputable and other than Iwano's (Iwano et al., 2002), cell tracking studies were negative (Humphreys et al., 2010, Hertig et al., 2008, Koesters et al., 2010). Activation of Wnt-β signalling involved in EMT has been reported in alveolar epithelial type II cells in animal models of pulmonary fibrosis and IPF patients, as well as in skin, liver, kidney and cardiac fibrosis (Wei et al., 2011, Baarsma et al., 2011). Recently, two groups reported evidence for EMT associated changes in SSc skin (Nakamura et al., 1995, Gillespie et al.,

2011), however, these reports were not followed by more extensive studies. EMT could link the changes found in the SSc epidermis, with the persistent myofibroblast phenotype seen in the underlying dermis.

### 1.4.5 Connective tissue growth factor (CTGF)

#### 1.4.5.1 Structure and function

CTGF, often used as readout of downstream TGF-β signalling, is a 36-38 kDa protein, a member of CCN family (Leask and Abraham, 2006) also known as CCN2. This molecule consists of four domains and a hinge region, which can be proteolyticaly cleaved to generate N- and C-terminal fragments. The C-terminal fragment can be additional cleaved to generate small C-terminal fragment containing domain 4. Domains of the C-terminal fragment regulate fibroblast proliferation, while the N-terminal fragment domains mediate collagen synthesis and myofibroblast differentiation (Grotendorst and Duncan, 2005). As shown in figure 1.9 the each domain interacts with different ligands. However some ligands can interact with more than one domain.

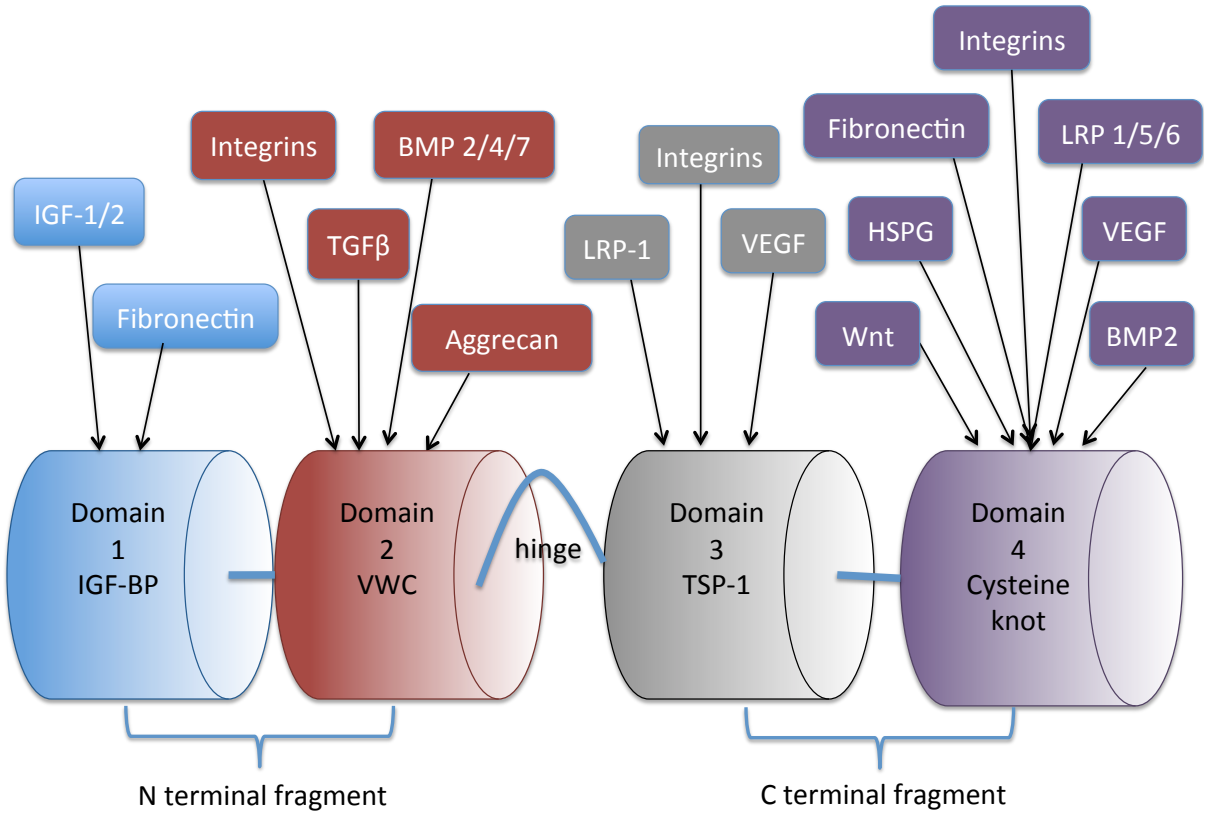


**Figure 1.9. CTGF structure and its binding partners.**

IGF-BP - insulin-like growth factor-binding protein domain; VWFC - von Willebrand factor type C repeat domain; TSP-1 - thrombospondin repeat type 1 domain; BMP - bone morphogenetic protein; HSPG - heparan sulphate proteoglycan; IGF - insulin-like growth factor; LRP - lipoprotein receptor-related protein; TGF-β - transforming growth factor-β; VEGF - vascular endothelial growth factor.

CTGF expression is mainly regulated at the transcriptional level. Its induction is mediated by TGF-β/Smad pathway via a Smad binding element within the proximal promoter (de Winter et al., 2008). CTGF is important for the formation of connective tissues and angiogenesis during development (Ivkovic et al., 2003, Perbal, 2004), but also later in adult life, CTGF is involved in a wide range of cellular processes, including proliferation, migration, adhesion, matrix production, and wound healing (Shi-Wen et al., 2008). The schematic of main signalling pathways directly stimulated by CTGF are shown in Figure 1.10.

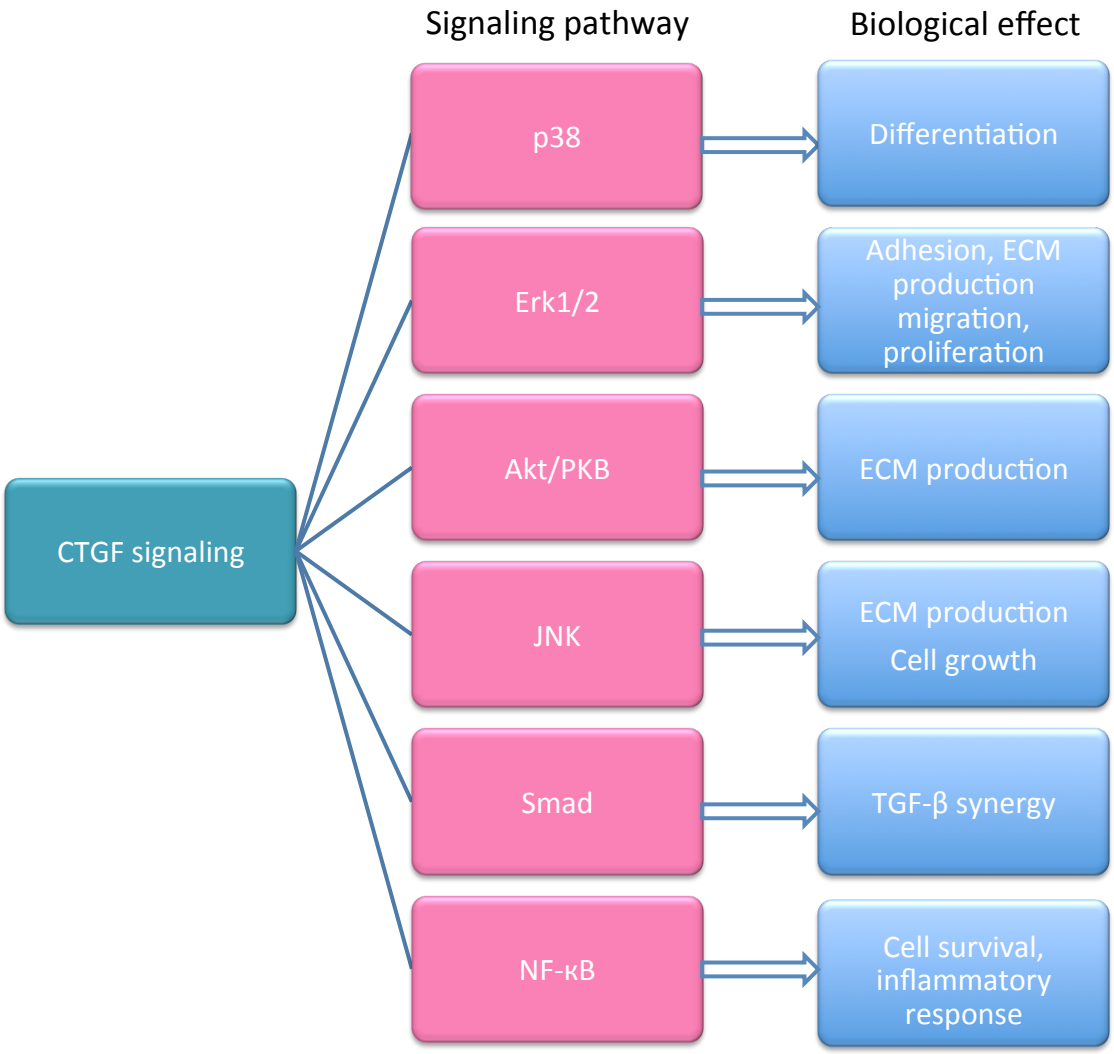


**Figure 1.10. Signalling pathways and biological actions directly stimulated by CTGF.**

#### 1.4.5.2 CTGF in fibrosis

CTGF has been shown to be up-regulated in excessive scaring and various fibrotic diseases, including pulmonary (Lasky et al., 1998, Pan et al., 2001), liver (Gressner et al., 2006, Kovalenko et al., 2009) and cardiac (Kemp et al., 2004) fibrosis, as well as SSc. In SSc polymorphisms in the CTGF promoter region influence its transcription and expression, and were found to be associated with more severe disease (Kovalenko et al., 2009, Granel et al., 2010, Fonseca et al., 2007). Several reports emphasized the important pathophysiological role of CTGF in SSc. Elevated levels of serum CTGF compared to controls were described in diffuse SSc, and linked to pulmonary complications (Sato et al., 2000). The highest CTGF levels were found in patients within 3 years of disease onset, and therefore suggest the role of CTGF as a marker of on-going fibrosis. Another study has found that plasma and dermal interstitial fluid levels for N-CTGF are significantly elevated in SSc and correlate with severity of skin disease (Dziadzio et al., 2005). Surprisingly, the study did not found significant changes in the levels of full-length CTGF or C-CTGF.

In SSc, CTGF overexpression is reported to be present in the fibrotic skin in early and established disease (Igarashi et al., 1996), in contrast to TGF-β1 mainly up-regulated early in the disease (Higley et al., 1994). This suggests, that CTGF not only mediates many of the downstream effects of TGF-β signalling, but can also promote fibrosis independently of TGF-β. Consistent with that, stimulation of normal fibroblasts with TGF-β induces CTGF via Smad3 binding site located within CTGF promoter. However, no reduction in CTGF promoter activity is observed in SSc dermal fibroblast with mutated Smad binding site. The SSc fibroblasts were reported to constitutively overexpress CTGF, which mRNA and protein levels are increased despite the absence of TGF-β (Denton and Abraham, 2001). The maintenance of the fibrotic phenotype in SSc fibroblasts was shown to be Smad independent, but instead dependent on ET-1 and SP1 (Holmes et al., 2003, Holmes et al., 2001, Shi-wen et al., 2007).

Another study went further and proved that CTGF is not only responsible for maintenance of fibrosis, but also development of fibrotic changes. It reported that mice with fibroblast-specific expression of CTGF develop a spontaneous fibrogenic phenotype, including thickening of the dermis, without prior TGF-β stimulation (Sonnylal et al., 2010).

Other examples of CTGF pro-fibrotic properties, include association of increased CTGF mRNA and protein level with myofibroblast accumulation and collagen deposition in radiation fibrosis (Vozenin-Brotons et al., 2003), and protection from liver fibrosis with CTGF siRNA (Li et al., 2006). Animal studies with tight skin mouse and bleomycin induced fibrosis models have confirmed the importance of CTGF in the development of fibrosis (Bonniaud et al., 2004, Liu et al., 2010).

## 1.5 Inflammation

Inflammation is an adaptive process triggered by infection, tissue injury or tissue stress in order to eradicate microbial agents, repair damaged tissue, and restore homeostasis (Fig.1.11). The process is characterized by vasodilation of the local blood vessels and increased blood flow, increased permeability of the capillaries leading to leakage of fluid into the interstitial space; migration of granulocytes and monocytes into the tissue, and swelling. Responsible for these reactions are immune cells, local cells e.g. keratinocytes and endothelial cells, as well as chemical mediators, released from injured or activated cells to co-ordinate the development of the inflammatory response. These mediators include: complement, coagulation and fibrinolytic systems reaction products, neuropeptides (kinins and tachykinins), eicosanoids, platelet activating factor, histamine, serotonin (5-HT), prostaglandins, nitric oxide, cytokines (e.g. IL-1 and IL-6) and chemokines.

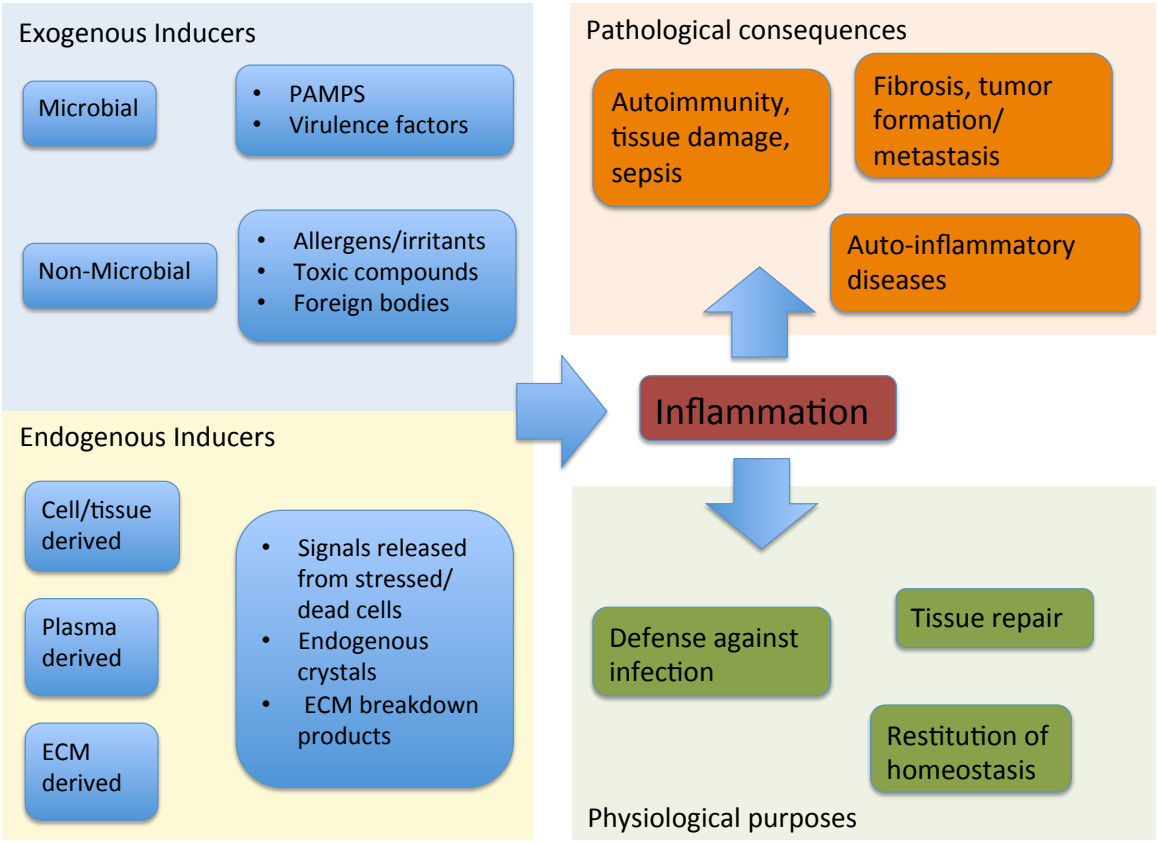


**Figure 1.11. Inducers and possible outcomes of inflammation.**

### 1.5.1 Types of inflammation

There are three distinct phases of inflammatory responses: an acute transient phase, characterized by local vasodilation and increased capillary permeability, followed by a delayed, sub acute phase, characterized by infiltration of leukocytes and phagocytic cells, and a chronic proliferative phase, with tissue degeneration and fibrosis. Redness, swelling, heat and pain are hallmarks of acute inflammation, which in normal conditions resolves itself. However, if the stimulus triggering inflammation persists it leads to chronic inflammation and disease. The production of growth factor, proteases and reactive oxygen species (ROS) by neutrophils and macrophages results in tissue damage, fibroblast proliferation, collagen accumulation and fibrosis. Chronic inflammation is characterised by simultaneous inflammation and repair processes, regulated by an interaction between cytokines promoting inflammatory cell recruitment/activation, and

growth factors that promote healing. The main differences between acute and chronic inflammation are summarised in the table below (Table 1.3).

**Table 1.3 Differences between acute and chronic inflammation.**

| Features | Acute Inflammation | Chronic Inflammation |
|---|---|---|
| Onset | Rapid | Delayed |
| Duration | <3 weeks) | >6 weeks |
| Cause | • Physical/chemical insult<br>• Pathogen<br>• Tissue necrosis | • Persistent infection<br>• Presence of a foreign object<br>• Autoimmunity |
| Main type of cells involved | • Neutrophils<br>• Macrophages | • Lymphocytes<br>• Macrophages<br>• Fibroblasts |
| Edema | Present | Absent |
| Angiogenesis | Absent | Present |
| Fibrosis | Absent | Present |
| Pathogenic processes | • Increased blood flow<br>• Increased capillary permeability<br>• Neutrophils migration<br>• Recruitment and activation of leukocytes | • Macrophage and lymphocytes infiltration<br>• Tissue damage (ROS, hydrolytic enzymes and inflammatory responses)<br>• Angiogenesis<br>• Granuloma formation<br>• Fibrosis |

Additionally, the inflammation might be also divided into microbial induced and sterile. During the microbial induced inflammation, the innate immune system is involved in an early response. Highly conserved pathogen associated molecular patterns (PAMPs) or virulence factors of bacteria, fungi or viruses, become recognised by pattern recognition receptors (PRRs), expressed on innate immune cells such as dendritic cells, macrophages and neutrophils. A wide range of PPRs was discovered over the time consisting of:

- toll like receptors (TLRs) - transmembrane proteins located at the cell surface or in endosomes,
- RIG-I-like receptors (RLRs) - intracellular proteins primarily involved in antiviral responses,
- NOD-like receptors (NLRs) – cytoplasmic receptors,
- C-type lectin receptors (CLRs) – trans-membrane receptors characterized by the presence of a carbohydrate binding domain,
- absence in melanoma 2 (AIM2) -like receptors- characterized by the presence of a pyrin domain and a DNA binding HIN domain, involved in the detection of intracellular microbial DNA.

Following ligand binding, these receptors rapidly induce host innate immune responses and activate downstream signalling pathways, such as NF-κβ, MAPK and IFN type I pathways, that culminate in the induction of inflammatory responses mediated by various cytokines and chemokines, which subsequently leads to the eradication of the pathogen.

Sterile inflammation typically occurs in the absence of microorganisms and is induced as a result of trauma, ischaemia-reperfusion or chemical injury. Typical examples of sterile inflammation are asbestosis - where chronic inhalation of asbestos leads to persistent activation of alveolar macrophages and result in pulmonary interstitial fibrosis, and atherosclerosis, where cholesterol crystals engulfed by macrophages cause the activation and recruitment of inflammatory cells, endothelial cell dysfunction and plaque formation. As in microbial induced inflammation, during sterile inflammation the recruitment of neutrophils and macrophages and the production of pro-inflammatory cytokines and

chemokines (particularly TNF and IL1) occur. However, the PPRs instead of recognizing PAMPs, bind Damage-Associated Molecular Patterns (DAMPs), host derived non-microbial stimuli, which are released following tissue injury or cell death.

### 1.5.2 Damage-Associated Molecular Patterns (DAMPs).

DAMPs also known, as alarmins are endogenous factors that are under normal physiological conditions present intracellularly, and thus hidden from the immune system. However, during cellular stress or injury, they become released into the extracellular environment by dying cells and become available to bind PPRs, and trigger sterile inflammation. Classical example of intracellular DAMPs are released from necrotic cells chromatin associated protein high-mobility group box 1 (HMGB1), heat shock proteins (HSPs), and purine metabolites such as ATP12 and uric acid. S100A8 and S100A9 are other examples of intracellular DAMPs that in physiological conditions have a role in migration and cytoskeletal metabolism. However, cell damage or activation of phagocytes triggers their release into the extracellular space, where they become danger signals that activate immune cells and vascular endothelium. Additionally, DAMPs can be located extracellularly and become released from damaged ECM during tissue injury. The ECM fragments, such as hyaluronan, heparan sulphate and biglycan, are generated by proteolytic degradation of ECM, by enzymes released from dying cells or proteases promoting tissue repair and remodeling. Figure 1.12 below represents the schematic of DAMPs signal transduction pathway.

Increased serum levels of DAMPs have been associated with many inflammatory diseases, including arthritis, atherosclerosis, Crohn's disease and cancer (Pullerits et al., 2008, Brezniceanu et al., 2003, Berntzen et al., 1989, Cesaro et al., 2012). Therapeutic strategies are being developed to regulate the expression of these DAMPs for the treatment of these diseases.

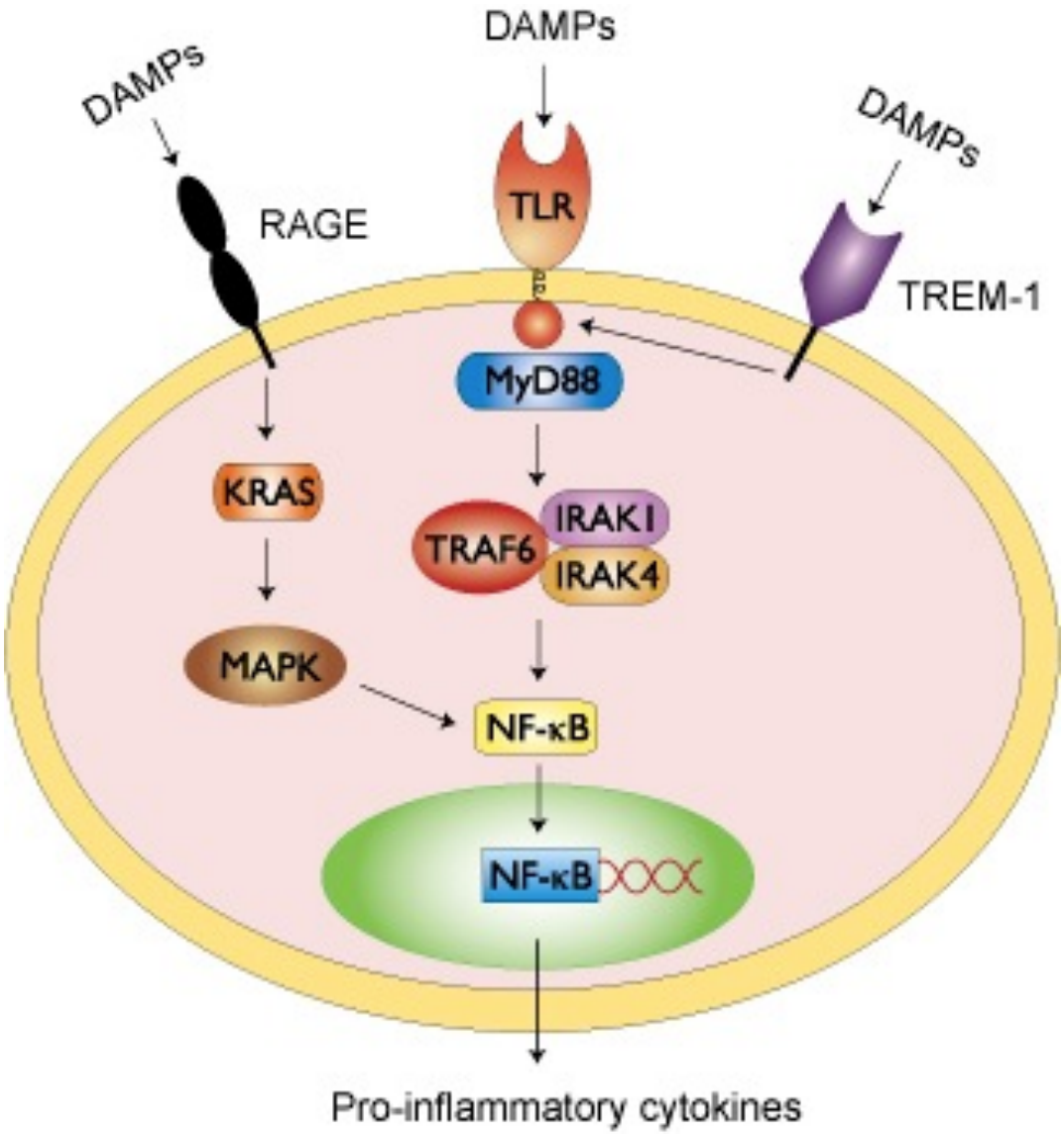


**Figure 1.12. DAMPs signalling.**

(From www.invivogen.com)

### 1.5.3 Skin as immune organ

Skin, as the first line of defense against microbes and physical, chemical and mechanical damage, has evolved complex immune-surveillance mechanisms that control tolerance and immune responses in the skin. The main resident-immune cells in the epidermis are Langerhans cells, but T cells (mainly CD8+) can also be found. Whereas the dermis contains many specialized immune cells, including macrophages, DCs, CD4+ T helper cells, γδ T cells and natural killer T cells, as well as mast cells and fibroblasts. Dermis also comprises lymphatic and vascular canals, enabling cell migration.

Barrier disruption e.g. due to UV light, irritants or infection, triggers a coordinated immune response to maintain skin homeostasis (Fig. 1.13). Although, resident immune cells are essential for re-establishing homeostasis, they can also contribute to tissue pathology such as fibrosis. Epidermal Langerhans detect danger signals in the skin however; keratinocytes have also this ability, and upon stimulation produce pro-inflammatory cytokines, which activate dermal DCs and plasmacytoid DCs (pDCs). Activation of pDCs leads to release of IFN-α and therefore, further stimulation of dermal DCs, which leads to activation, and clonal expansion of resident memory CD4+ and CD8+ T cells. In turn, T cell release pro-inflammatory cytokines and chemokines, further stimulating epithelial and mesenchymal cells, and thus magnifying the inflammatory response. In addition, resident T cells migrate into the epidermis and engage in an immune–epithelial cell cross talk. Fibroblasts contribute to the local inflammatory response by producing TNF and IL-6, while natural killer T cells produce TNF and IFN-γ.

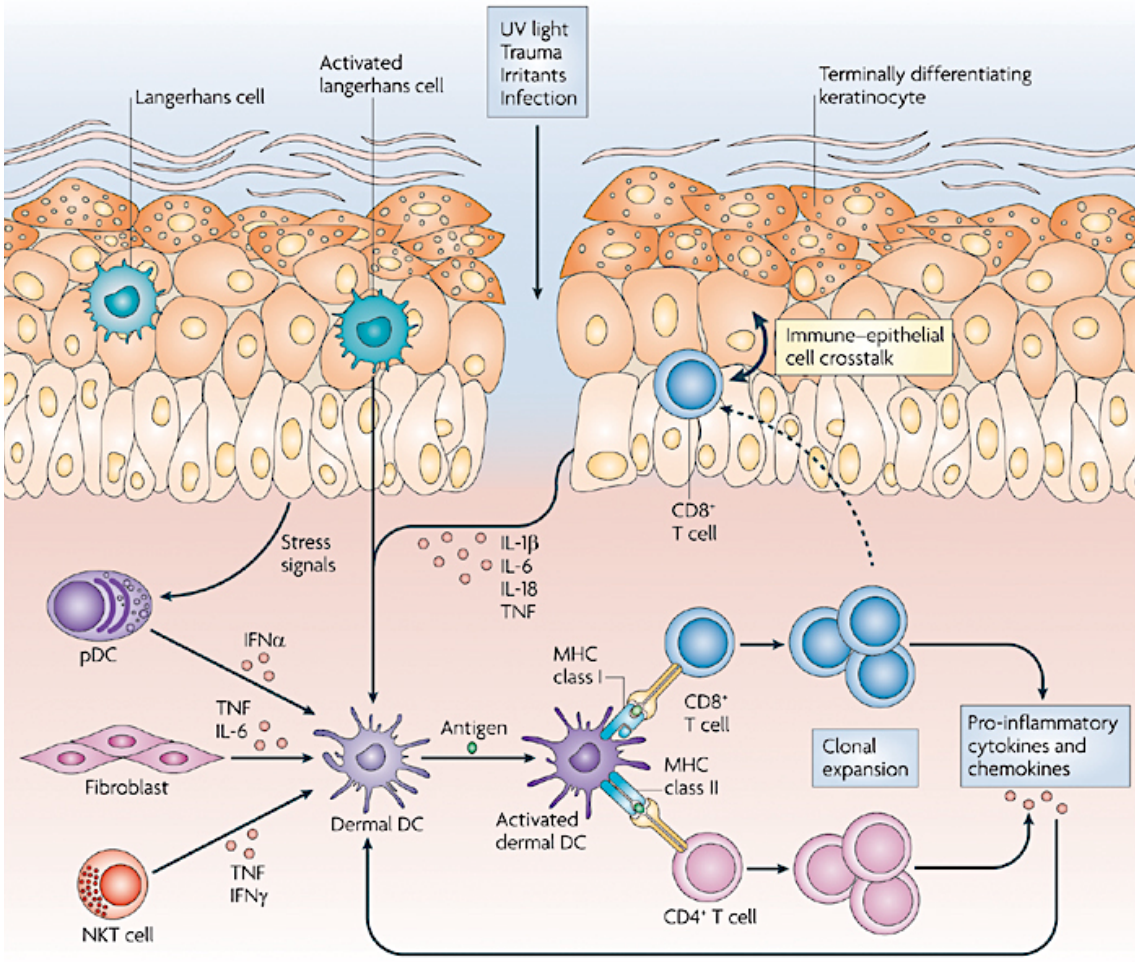


**Figure 1.13 Skin immune response.**

(From Nestle et al., 2009)

Historically, the epidermis was considered to be just a passive mechanical barrier that forms surface of the body, preventing microbes from entering deeper tissues and fluid loss. The sources of cytokines in the skin were thought to be limited to dermal dendritic cells, epidermal Langerhans cell, melanocytes, and migrating lymphocytes. However, keratinocytes are also major contributors to epidermal cytokine production (Grone, 2002). Since 1970s reports started to emerge that challenged the view of immunologically passive epidermis and prove that the keratinocytes are actively taking part in inflammatory response and are capable of producing a number of cytokines, chemokines, neuropeptides and DAMPs involved in immune responses to the stimuli (Barker et al.,

1991, Steinhoff et al., 2001). Such stimuli include ultraviolet radiation, allergens/irritants, microbiological agents or trauma/wound repair.

### 1.5.3.1 Keratinocytes as immune cells

Keratinocytes can recognize DAMPs and PAMPs due to a set of cell surface and endosomal receptors, including Fc receptors (Tigalonowa et al., 1990), complement receptors, mannose receptors (Szolnoky et al., 2001), TLRs 1–6 and 9 (Baker et al., 2003, Pivarcsi et al., 2003, Lebre et al., 2007), as well as NLRs (Harder and Nunez, 2009, Kobayashi et al., 2009) and RLRs (Kalali et al., 2008). Ligand binding to these receptors results in effective endocytosis and subsequent killing of microbiological agents (Kisich et al., 2007) and release of chemotactic mediators to recruit neutrophils, dendritic cells, monocytes and lymphocytes (Fig 1.14).

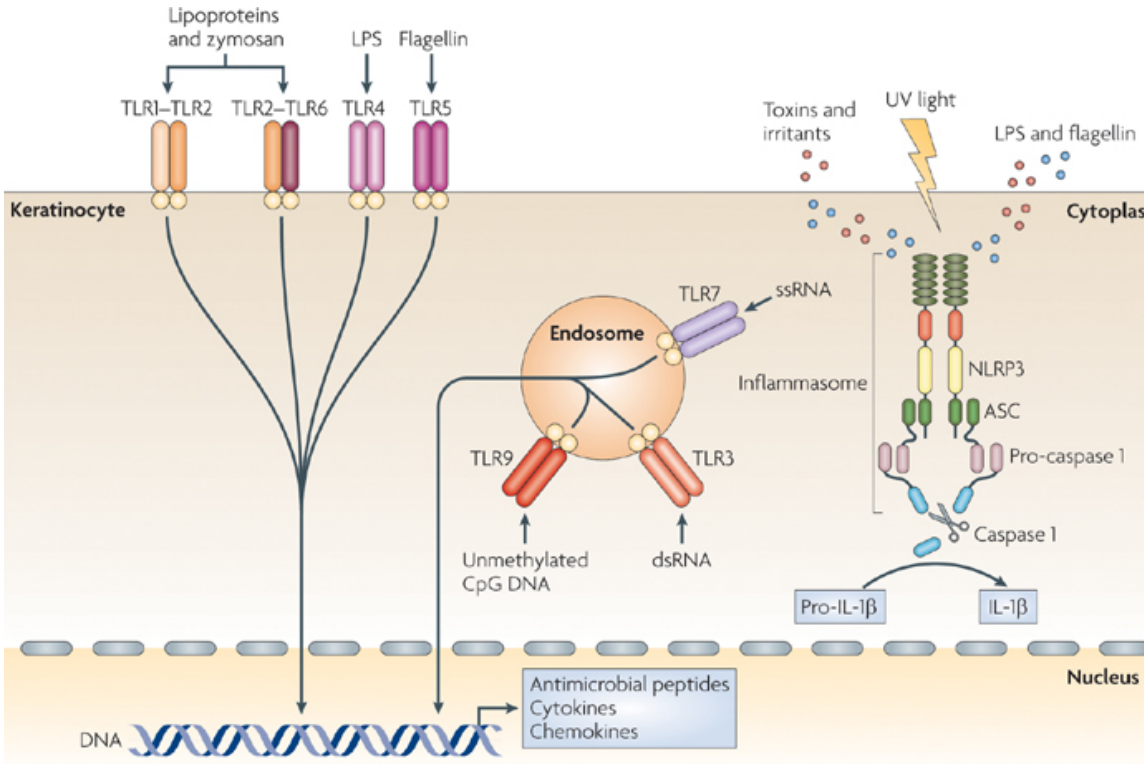


**Figure 1.14 Keratinocytes as sensors of exogenous and endogenous inducers of inflammation**. (From Nestle et al. 2009)

Moreover, studies on keratinocytes exposed to *Staphylococcus aureus* suggest that keratinocytes are able to directly stimulate T-cells (Lebre et al., 2007, Strange et al., 1994, Ardern-Jones et al., 2007) by acting as non-professional antigen presenting cells. They usually express low levels of co-stimulatory molecules CD80/86, and class-II–mediated presentation of antigen by keratinocytes results in tolerance or anergy of T cells (Bal et al., 1990, Gaspari et al., 1988), rather than T cell activation. However, in multiple human skin diseases keratinocytes can up-regulate both major histocompatibility complex class I and II molecules (Banno et al., 2003, Bieber et al., 1989, Wittmann et al., 2005), and during inflammation keratinocytes may be able to stimulate CD4+ and CD8+ memory T cells (Black et al., 2007, Ardern-Jones et al., 2007, Mutis et al., 1993, Wittmann et al., 2005). IFN-γ was shown to up-regulate MHC class II expression on primary human keratinocytes and keratinocyte cell lines in vitro (Nickoloff and Naidu, 1994). Moreover, it has been shown that apart from the classical presentation of peptides, keratinocytes can also use non-classical pathways for presentation of glycolipids via CD1 molecules overexpressed in psoriatic skin or after mechanic trauma (Bonish et al., 2000).

Keratinocytes can also indirectly influence immune responses, by releasing cytokines such as, IL-1, IL-6, IL-10, IL-18 and TNF (Albanesi et al., 2005). Particularly important, is the production of IL-1, a pleiotropic cytokine with a wide range of biological effects, including activation of T helper cells and DCs, and the promotion of maturation and clonal expansion of B cells (Arend et al., 2008). In healthy skin, keratinocytes constitutively synthesize IL-1α and IL-1β, which following keratinocyte activation, are released in biologically active form. Keratinocytes also promote differentiation of monocytes into DCs via release of GM-CSF (Pastore et al., 1997). Moreover, keratinocytes are an important source of chemokines modulating an immune response by attracting immune cells into the skin. Expression of CC-chemokine ligands (CCL): CCL20, CXCL9, CXCL10 and CXCL11 from activated keratinocytes selectively attracts effector T cells to the skin and was reported in several diseases, characterized by T cell infiltration, like psoriasis (Albanesi et al., 2005).

Furthermore, expression of CCL20 by keratinocytes regulates the trafficking of Langerhans cell precursors to the epithelium (Dieu-Nosjean et al., 2000). Upon activation, keratinocytes are also able to recruit neutrophils by releasing IL-8, as it was demonstrated in the inflamed epidermis of patients with psoriasis. Additionally, damaged keratinocytes are an important source of antimicrobial peptides (AMPs), in particular β-defensins and cathelicidins. These peptides not only directly kill the pathogen, but also recruit immune cells and increase production of cytokines (Gilliet and Lande, 2008, Lai and Gallo, 2009). The production of AMPs by keratinocytes is regulated by T cell-derived cytokines, mainly IL-17A and IL-22 (Kolls et al., 2008).

Over the decades, keratinocytes have been shown to be pro-inflammatory effector cells that react to damaging insults by the coordinated production of a wide range of AMPs, pro-inflammatory cytokines and chemokines and their receptors (Table 1.4) (Grone, 2002, Uchi et al., 2000). The release of these factors by keratinocytes factors has multiple consequences for the migration of inflammatory cells, systemic effects on the immune system, but also influences keratinocyte proliferation and differentiation processes, and affects the production of other cytokines by keratinocytes. What is more, release of cytokines from activated keratinocytes was shown to precede any inflammatory cell influx into the area and reflects changes intrinsic to the epidermis (Nickoloff and Naidu, 1994), suggesting that epidermal keratinocytes could function as initiators of cutaneous inflammation. Moreover, mice with deletion of c-Jun and JunB in keratinocytes, spontaneously develop chronic inflammation in the skin and joints (Zenz et al., 2005). Similar cutaneous inflammation was observed in animals with deletion of IκB kinase-β (IKKβ), a regulatory kinase of NF-κB pathway in keratinocytes (Pasparakis et al., 2002). This demonstrates that genetic modification of key signalling pathways involved in inflammatory immune responses in keratinocytes can alter skin homeostasis and induce immunopathology, thus supporting a crucial role for keratinocytes in regulating systemic immune responses.

**Table 1.4. Keratinocytes as source and target of cytokines.**

| Cytokine/ Chemokine | Main functions in skin | Receptor | Receptor mediated effect |
|---|---|---|---|
| IL-1a, b | Pro-inflammatory | IL-1R1/R2 | Cytokine release |
| IL-1Ra | Competitive IL-1 inhibitor | IL-1R1/R2 | Prevents cytokine release |
| IL-6 | Pro-inflammatory, keratinocytes proliferation | IL-6R | Growth induction |
| IL-10 | Immunomodulatory | IL-10R | Th1 inhibition, B cell proliferation, antibody production |
| IL-7, IL-15 | T-cell trophic | - | - |
| IL-8 | Pro-inflammatory, neutrophil attraction | CXCR2 | Attract neutrophils |
| IL-12, IL-18 | Immunomodulatory | IL-18R | |
| IL-20 | Keratinocytes proliferation and differentiation | - | - |
| TNF-a | Pro-inflammatory, induce adhesion molecules | TNF-a receptors | Cytokine release, ICAM-1 induction |
| M-CSF | Monocytes and macrophages proliferation, differentiation, and survival | - | - |
| GM-CSF | Hematopoietic growth factor and immune modulator | - | - |
| TGF-a | Enhance keratinocytes motility | TGF-b receptors | Differentiation control |
| TGF-b1 | Inhibit keratinocytes growth | | |
| CCL2/MCP-1 | Inflammatory attractant | - | - |
| CCL20 | B cell maturation, migration and recruitment of DC, LC and T cells | CCR6 | B cell maturation, migration and recruitment of DC, LC and T cells |
| HB-EGF | Keratinocytes growth factor | EGF-R | Growth induction |
| FGF-2, FGF-10 | Keratinocytes growth factor | FGFR2 | Growth induction |
| IGF-1/IGF-2 | Keratinocytes growth factor | IGF-1R | Growth induction |

| | | | |
|---|---|---|---|
| NGF | Keratinocytes and melanocyte growth factor | NGF receptors | Growth induction |
| VEGF | Endothelial cell growth factor | - | - |
| PDGF | Fibroblast and smooth muscle cell growth factor | - | - |
| ET-1 | Keratinocytes and melanocyte growth factor, vasoconstrictor | ET-1 receptors | Growth induction |
| IFN-b | Defense against viral infections | IFN-a/b receptor | Growth inhibition |
| IFN-g | Th1 induction, | IFN-g receptor | Induce HLA-DR and ICAM-1 |
| CXCL9/ CXCL10/11 | Inflammatory mediator, T cell attractant | - | - |
| CXC12 | T-lymphocytes, monocytes and LC attractant | - | - |
| - | - | IL-2R | Sensitize keratinocytes towards other growth inhibitory molecules |
| - | - | IL-4R | Keratinocytes proliferation, IL-6 production |
| - | - | IL-13R | IL-6 production |
| - | - | IL-17R | Modulate effects of IL-4 and IFNg on keratinocytes activation |
| - | - | IL-20R | Keratinocytes proliferation |
| - | - | LIF-R | Cellular differentiation, proliferation and survival |

### 1.6 S100 calcium binding protein A9 (S100A9)

S100A9 belongs to a family of 20 distinct calcium (Ca2+) binding protein and is one of DAMPs (Foell et al., 2007). This small protein with molecular weight of 14kDa is distinguished from other S100 proteins by its long and flexible C-terminal region (Itou et al., 2002). Crystal structure of each monomer consists of the hinge region that connects two EF-hand motifs. High affinity Ca2+ binding site is located at the C-terminus and a low-affinity Ca2+ binding site at the N-terminus. Upon Ca2+ binding there is a conformational change enabling macromolecular interactions. S100A9 can form homo-dimers (e.g. cancer cell lines including breast MCF7 and hepatocellular SK-Hep-1), or hetero-dimers and tetramers with S100A8 (Korndorfer et al., 2007). Its activity is dependent on the extracellular Ca2+ and zinc (Zn2+) concentrations. Depending on the subunit composition, it can have different biological activity (Newton and Hogg, 1998).

S100A9 was first discovered in 1987, in the infiltrating macrophages of rheumatoid arthritis (RA) patients (Odink et al., 1987). However, S100A9 protein was later re-discovered in several contexts, which is reflected in its complex nomenclature:

- NIF;
- CGLB;
- L1AG;
- MAC387
- Calgranulin B (CAGB)
- p14
- Myeloid related protein of molecular weight 14 kDa (MRP14)
- Migration inhibitory factor-related protein of molecular weight 14 kDa (MIF)
- CFAG
- LIAG
- 60B8AG

S100A9 is expressed in neutrophils, activated monocytes and macrophages, but its up-regulated expression was found in many inflammatory diseases at the site of

inflammation (Roth et al., 2003, Nacken et al., 2003) and in the serum of patients with RA (Liao et al., 2004), multiple sclerosis (Bogumil et al., 1998), Crohn's disease (Lugering et al., 1995), and connective tissue diseases such as SLE, SSc and Sjogren's syndrome (Kuruto et al., 1990). The protein is also associated with many different cancer types (non-small cell lung carcinoma gastric cancer, prostate cancer, pulmonary adenocarcinoma, squamous cervical cancer, prostate cancer, breast cancer) (Cross et al., 2005). Over expression of S100A9 in non-small cell lung carcinoma is associated with poor prognosis (Kawai et al., 2011). Moreover, S100A9 is also used as the marker of epithelial cells activation.

### 1.6.1 S100A9 in keratinocytes

Barely detectable in healthy epidermis, S100A9 expression was shown to be strongly induced during treatments simulating environmental stress situations such as mechanical stress (tape stripping), chemical stress (SDS and vaseline application), and UV light exposure (solar-simulated radiation) (Marionnet et al., 2003). Furthermore, S100A9 is up-regulated during wound healing (Thorey et al., 2001) and in hyper-proliferative psoriatic keratinocytes (Broome et al., 2003).

Interestingly, evidence from activin-overexpressing mice, which develop a hyper-proliferative and abnormally differentiated epidermis in the absence of inflammation, suggests that S100A9 overexpression in wound keratinocytes is most likely related to the activated state of the keratinocytes and not secondary to the inflammation of the skin (Munz et al., 1999, Thorey et al., 2001). There are several findings supporting the expression of S100A9 and S100A8 in keratinocytes being involved in epithelial differentiation and linked to the imbalance between proliferation and differentiation.

The gene encoding S100A9 is located in the epidermal differentiation complex, within human chromosome 1q21 (Mischke et al., 1996), alongside genes encoding the cornified envelope precursors, involucrin, loricrin and several keratins. Such co-localization of

terminal differentiation genes with Ca2+ binding S100A9, points to importance of Ca2+ levels in control the differentiation of epithelial cells and the expression of genes encoding epidermal structural proteins, and suggests that expression of these genes is coordinately regulated (Hardas et al., 1996).

Additionally, *in vitro* studies on HaCaT and HNEK cells showed close correlation of keratinocyte S100A9 expression with degree of differentiation, suggesting involvement in terminal differentiation pathway (Martinsson et al., 2005, Voss et al., 2011). Its overexpression in diseased epidermis is often linked to keratinocyte hyper-proliferation and abnormal keratinocyte differentiation (Broome et al., 2003, Benoit et al., 2006). Furthermore, injury-regulated induction of S100A8 and S100A9 genes was reported in differentiating suprabasal wound keratinocytes during the hyper-proliferative phase. Interestingly, S100A9 is not only located subcellular associated with the keratin cytoskeleton, but can be also released into the culture media (Thorey et al., 2001, Nukui et al., 2008). This indicates that S100A9 might have various roles in different processes.

### 1.6.2 Pleiotrophy of S100A9

S100A9 has diverse functions, including involvement in the reorganization of the keratin cytoskeleton in the wounded epidermis, chemoattraction of inflammatory cells. The table 1.5 below contains more detailed information with key references. Previous studies indicate that S100A9 activity can be regulated at post-translational level and these modifications result in decreased inflammatory activity, for example S-nitrosylation reduces NADPH oxidase activation (Cheng et al., 2010), while S-glutathionylation reduces its capacity to hetero-dimarise with S100A8 and bind fibronectin, but doesn't affect arachidonic acid binding (Boyce and Ham, 1985). Also oxidation of S100A9 at methionine 63 and 83 was shown to regulate neutrophil migration (Sroussi et al., 2007).

**Table 1.5. Pleiotrophic effects of S100A9.**

| Function | References |
|---|---|
| Cell mobility | • S100A8/A9 regulates polymerisation of microtubules (Aden et al., 2010)<br>• In epithelial cells Ca-S100A8/A9 complexes bind to keratin intermediate filaments increasing cell mobility (Goebeler et al., 1995) |
| Profibrotic | • By sequestering zinc inhibits ECM degradation by MMPs (Rheinwald and Green, 1975)<br>• Stimulates fibroblast growth as monomer and homodimer (Shibata et al., 2004), however as heterodimer inhibits fibroblast growth (Yui et al., 1997)<br>• Activates human lung fibroblasts via RAGE mediated ERK1/2 MAP-kinase and NF-kB (Xu et al., 2013a) |
| Proinflammatory | • Activates NADPH oxidase (Kahan et al., 2009, Chen et al., 1985)<br>• Induces expression and secretion of pro-inflammatory cytokines such as IL-6, IL-8, IL-10, IL-1b, TNF-a, MCP-1 in RA model (Sunahori et al., 2006, Nukui et al., 2008);<br>• Important in development of autoreactive CD8+ T cells (Herfs et al., 2008)<br>• Recruits inflammatory cells during inflammation (Burwinkel et al., 1994, Hessian et al., 1993, Guignard et al., 1996, McNeill et al., 2007, Walter and Ron, 2011)<br>• Enhances trans-endothelial migration (Gerritsen et al., 2003)<br>• Regulates maturation of myeloid cells e.g. its production correlates with the surface expression of CD11b by monocytes and macrophages (Kerkhoff et al., 2002) |
| Regulation of differentiation and proliferation of keratinocytes | • As heterodimer stimulates keratinocytes growth(Rammes et al., 1997) or inhibits proliferation and stimulates differentiation (Voss et al., 2011) |
| Cancer promotion | • Inhibits immune response to cancer by recruitment of myeloid derived suppressor cells (MDSCs) and inhibition of dendritic cells (Cheng et al., 2008),<br>• S100A9 knockout improves rate of rejection of EL4 lymphoma (Connelly et al., 2010) |
| Other | • Bound to CD36 promotes fatty acid uptake (Kerkhoff et al., 2001)<br>• Activates NF-kB (Kahan et al., 2009), and the activation is TLR4 dependent (Averill et al., 2011); it also induces degradation of IkBa |

### 1.6.3 S100A9 signalling

As described above S100A9 has a whole array of functions. It is unsurprising, considering the various molecules it can bind. S100A9 bound to Ca2+ was reported as an agonist of a wide range of molecules including:

- Arachidonic acid
- Cytoskeletal elements (e.g. keratin filaments)
- Receptor for Advanced Glycosylation Endproducts (RAGE)
- Toll Like Receptor 4 (TLR4)
- The major fatty acid transporter CD36
- Matrix metallo-proteinases (MMPs)
- Fibronectin
- Heparin sulphate glycosaminoglycans

The affinity of binding might depend on the subunits, as in vitro studies show that homo-dimer has higher affinity than heterodimer S100A8/A9 to bind TLR4 and RAGE (Bjork et al., 2009).

Enhanced production of S100A9 was found to be related not only to injury, but also stimulation with LPS (Henke et al., 2006), TNFα, IL-1α (Hayashi et al., 2007), IL-6 (Lee et al., 2012), IL-22 (Boniface et al., 2005) and IL-17 (Liang et al., 2006), VEGF (Hiratsuka et al., 2006), TPA (Schlingemann et al., 2003). However, reports on the TGF-β effects on S100A9 are more conflicting. Hiratsuka after adding exogenous TGF-β observed increased levels of S100A9 in mice (Hiratsuka et al., 2006), while in vitro work of Hayashi showed suppression of mRNA upon stimulation of gingival keratinocytes with TGFβ (Hayashi et al., 2007). Furthermore, in mice lacking S100A9 and TLR4, down-regulation of TGFβ expression was reported (Kallberg et al., 2012). Suppression of S100A9 by and KGF was also described (Bando et al., 2010).

The downstream signalling leads to activation of MAPK, JAK/STAT, IKK/ NF-κβ, PKC pathways, resulting in activation of several transcription factors, which bind to S100A9

promoter AP-1(Zenz et al., 2005), NF-κβ, STAT3 (Cheng et al., 2008), PU.1 (Henkel et al., 2002), C/EBP (Hayashi et al., 2007, Klempt et al., 1998, Kuruto-Niwa et al., 1998) and triggers different responses.

A pharmacological compound quinoline-3-carboxamide (Q compound) was shown to bind the S100A9 homodimer limiting binding to TLR4-MD2, RAGE and arachidonic acid (Bjork et al., 2009). The drug is currently in phase II clinical trials for patients with metastatic prostate cancer (Pili et al., 2011, Dalrymple et al., 2012) and multiple sclerosis (Schulze-Topphoff et al., 2012). In February 2011, the oral version of Q compound called paquinimod (57-57, ABR-215757) was granted orphan medicinal product status, for the indication SSc and the phase I clinical trials started in 2012.

### 1.6.4 S100A9 in SSc and other fibrotic diseases

Several reports demonstrated pro-fibrotic potential of S100A9. S100A9 was shown to promote proliferation (Shibata et al., 2004) and activation of fibroblasts, as well as induce production of collagen type III and α-SMA in lung fibroblasts (Xu et al., 2013a). S100A9 was also shown to inhibit degradation of ECM by MMPs, which might lead to further accumulation of ECM (Isaksen and Fagerhol, 2001). Moreover, S100A9 can also perpetuate inflammation closely linked to fibrosis, by up-regulating expression of pro-inflammatory cytokines IL-6, IL-8, IL-1β (Cesaro et al., 2012) and attracting immune cells.

Although, the role of S100A9 in SSc is unknown recent publications suggest its importance in the development of disease. Overexpression of both S100A9 receptors, RAGE (Davies et al., 2009) and TLR4 (Bhattacharyya et al., 2013) was previously reported in SSc skin. Additionally, signalling via TLR4 was shown to augment TGF-β responses contributing to fibrosis in SSc (Bhattacharyya et al., 2013). Furthermore, increased levels of S100A9 were found in SSc skin and plasma, especially in patients with lung or kidney involvement (Xu et al., 2013b, van Bon et al., 2014). Interestingly, S100A9 correlates closely with disease severity auto-autoantibodies levels.

## 1.7 Aims

Systemic sclerosis is a disease of complex pathophysiology, with interaction between many different cell types. In particular, fibroblasts are considered to be the major contributor to the disease pathogenesis. However, some the initiating events could occur within the most exposed part of the body, epidermis. Therefore the role of injured keratinocytes in SSc and their responses must be elucidated more clearly. Most SSc patients develop skin fibrosis and skin disease, which still accounts for significant morbidity in SSc despite the treatment improvements. An epidermal response to environmental triggers in SSc is likely to lead to activation of keratinocytes, which would affect they differentiation, and change in secreted growth factors and cytokines, causing abnormal cross talk with other cell types, especially fibroblasts. This thesis addresses the hypothesis that SSc epidermis plays an important role in propagation of skin fibrosis by releasing pro-inflammatory mediators and stimulating underlying fibroblasts

## 1.8 Experimental approach

To test this hypothesis analysis of epidermal blister sheets, dermal biopsies and immortal keratinocyte cell line has been employed:

- To characterize SSc epidermis and determine whether SSc epidermal keratinocytes have altered differentiation program,
- To examine evidence of SSc keratinocytes contribution to skin disease via process of epithelial to mesenchymal transition,
- To evaluate the secretome of SSc skin, separate for epidermal and dermal component.
- To investigate the effects of altered SSc keratinocyte derived mediators on underlying dermal fibroblast.

# Chapter Two

# Materials and Methods

## 2.1 Clinical material

All SSc patients used in this thesis fulfilled the American College of Rheumatology (ACR) preliminary criteria for disease and were classified into dcSSc and lcSSc according to internationally accepted criteria (LeRoy et al., 1988). Samples were classified as early SSc if within the first 2 years of disease onset, defined by the appearance of the first non-Raynaud's symptom. Patients with disease duration above 2 years were classified as established.

The clinical and laboratory parameters used for assessment of disease included: duration of disease, skin score, internal organ involvement, and serology. The extend of skin involvement was classified using mRSS total skin thickness score (Clements et al., 1995). The internal organ classification was used as previously described (Shand et al., 2007). All patients and healthy volunteers have given written informed consent to participate in the study.

### 2.1.1 Dermal punch biopsies

Punch biopsies of 4mm diameter were taken from forearm skin of healthy controls and SSc patients at various stage of disease. Tissue was used in an explant experiment (see 2.2.1) or fixed in formalin, embedded in wax and sectioned prior to staining.

### 2.1.2 Epidermal suction blisters

Suction blister cup (Ventipress, Uppsala, Sweden) was applied to the forearm as described previously (Rhodes et al., 1999). Under a pressure of 275–325 mmHg for approximately 2 hours, small blisters were raised (Fig 2.1), from which interstitial fluid was aspirated using an 18-gauge needle on a 2ml syringe (Kool et al., 2007), and frozen immediately at -70°C prior to analysis of S100A9 levels. The epidermal sheet (blister roof) was then carefully dissected with a scalpel and fine forceps, rinsed in phosphate-

buffered saline (PBS) (Life Technologies, Glasgow, UK) and immediately placed in RNA stabilization reagent RNAlater (Life Technologies). Samples were then stored overnight at 4°C, followed by longer-term storage at -70°C prior to RNA extraction.

**Figure 2.1. Suction pump and raised suction blister.**

The controls were recruited among primary Raynaud's syndrome patients and healthy people. Clinical details of the patients and controls involved in the study are listed in a table 2.1.

**Table 2.1. Clinical details of patients and controls.**

| | DcSSc | LcSSc | Controls |
|---|---|---|---|
| **N** | 24 | 8 | 9 |
| **Age (mean ± SD)** | 54.4 ± 7.48 | 59.3 ± 10.19 | 38.6 ± 6.52 |
| **Sex (Female/Male)** | 18/6 | 7/1 | 8/2 |
| **Stage of disease (early/established)** | 3/21 | 0/8 | NA |
| **Skin score (mRSS) (mean ± SD)** | 21.4 ± 10.46 | 8 ± 5.13 | NA |
| **Auto-antibodies** | | | |
| **Anti-topoisomerase-1 (ATA)** | 30.4% | 12.5% | NA |
| **Anti-nuclear (ANA)** | 82.6% | 100% | NA |
| **Anti-centromere (ACA)** | 4.3% | 25% | NA |
| **Anti-RNA polymerase (ARA)** | 17.4% | 0% | NA |
| **Anti-fibrillarin (U3RNP)** | 4.3% | 0% | NA |
| **Internal organ involvement** | | | |
| **Pulmonary** [a] | 47.8% | 37.5% | NA |
| **GI** [b] | 8.7% | 62.5% | NA |
| **Cardiac** [c] | 8.7% | 0% | NA |
| **None** | 8.7% | 25% | NA |

N - number of participants, SD – standard diviation, NA- non applicable; Skin score (mRSS) – assessment of skin thickening by palpation of the skin in 17 areas of the body (fingers, hands, forearms, arms, feet, legs and thighs, face, chest and abdomen) using a 0–3 scale, where 0 = normal, 1 = mild thickness, 2 = moderate thickness and 3 = severe thickness. Total skin score can range from 0 (no thickening) to 51 (severe thickening in all 17 areas). [a] defined as predicted forced vital capacity (FVC) or carbon monoxide diffusing capacity ($DL_{CO}$) of <55% or a 15% decline from baseline in FVC or $DL_{CO}$, with fibrosis confirmed on high-resolution CT. [b] defined as at least 3 episodes of intestinal pseudo-obstruction requiring hospitalization or requiring ≥6 weeks of enteral or parental nutritional support. [c] defined as haemodynamically significant cardiac arrhythmias, pericardial effusion or congestive cardiac failure requiring specific treatment in the absence of other known cardiac causes.

## 2.2 Cell culture

### 2.2.1 Skin explants culture for conditioned media

Forearm punch biopsies of 4mm diameter were taken from 12 healthy controls and 12 SSc patients. Their clinical details are listed in a table below.

**Table 2.2. Clinical information of patients and controls used in the conditioned media study.**

| | SSc patients | Control |
|---|---|---|
| **N** | 12 | 12 |
| **Age (year, mean ± SD)** | 53 ± 12.16 | 35.7 ± 11.00 |
| **Sex (female/male)** | 8/4 | 8/4 |
| **Stage of disease (early/established)** | 6/6 | NA |
| **Diffuse/ limited** | 9/3 | NA |
| **Auto-antibodies** | | |
| **Anti-topoisomerase-1 (ATA)** | 41.7% | NA |
| **Anti-nuclear (ANA)** | 83.3% | NA |

N - number of participants, SD – standard diviation, NA- non applicable

Biopsies were washed in PBS and incubated with trypsin-EDTA (Life Technologies) for 2hr at 37°C in 5% $CO_2$. Following extensive washing with PBS, epidermis was separated from the dermis along the basement membrane using forceps. Explants were then incubated overnight with 1ml of serum free media supplemented with penicillin streptomycin (P/S); dermis in high glucose Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) while epidermis in keratinocyte growth media (Life Technologies). Finally, conditioned media was aliquoted and stored at -80°C prior to analysis using Luminex or ELISA (Reiss et al., 2009, Beirne et al., 2009).

### 2.2.2 Human keratinocyte cell line (HaCaT)

HaCaT is a spontaneously immortalized human keratinocyte cell line developed through long-term culture of normal human adult skin keratinocytes at reduced calcium concentration and elevated temperature (Boukamp et al., 1988). It was used in this thesis as in contrast to many virally transformed keratinocyte cell lines, HaCaT is capable of normal differentiation including, expressing of involucrin and filaggrin (Boukamp et al., 1988). Moreover, although immortalized, HaCaT do not display invasive properties in vivo (Boukamp et al., 1988, Boukamp et al., 1985).

HaCaT (ATCC, Teddington, UK) cell cultures were established from departmental liquid nitrogen stocks and expanded in growth in DMEM supplemented with 10% fetal calf serum (FBS) (Life Technologies). In short, vials containing frozen cells (passage 10-12) were warmed in water bath at 37°C. Thawed cells were transferred to a 15 ml tube with 9 ml of warm media added, and centrifuged at 300 x g for 5 min. Supernatant was then removed and cells re-suspended in 10mls of growth media and seeded in to T75cm$^2$ flasks. The culture media was replaced every 2-3 days, and cells were passaged at 60-70% confluence. Cultures were inspected using a phase-contrast microscope to confirm typical cobble shape like keratinocytes morphology (Fig.2.2).

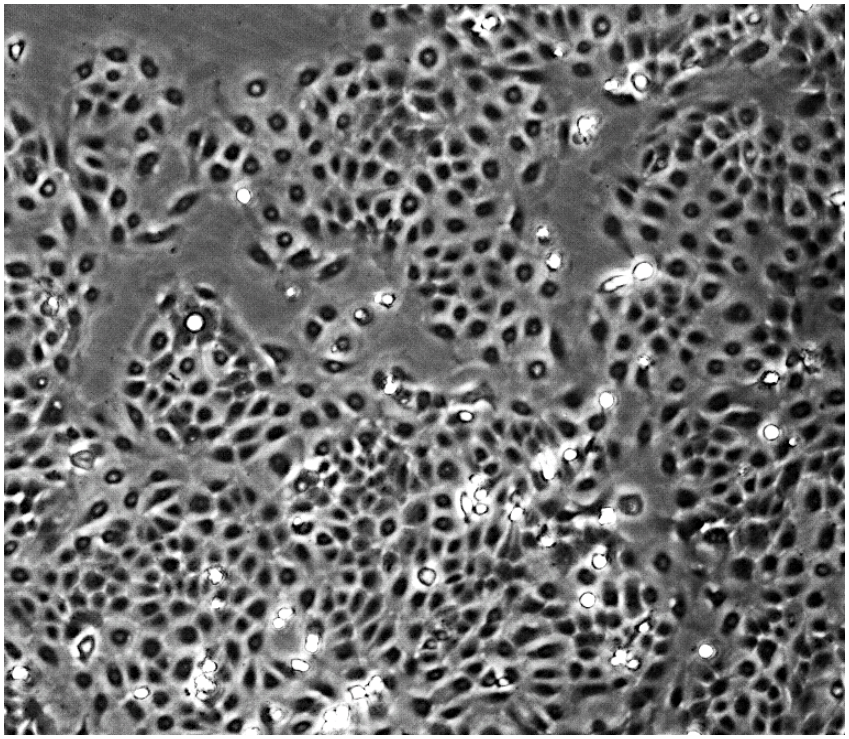

**Figure 2.2. Phase-contrast photomicrographs of HaCaT cells.**

### 2.2.3 Primary fibroblast strains

Dermal fibroblasts were grown from explant cultures of human forearm skin biopsies (4mm) in DMEM supplemented with 10% FBS and 100U/ml penicillin and 100μg/ml streptomycin (P/S) (Life Technologies). Under aseptic conditions in a laminar flow cabinet, the tissue was washed, finely cut, adhered and cultured in 25cm$^2$ tissue culture flasks in a humidified atmosphere of 5% $CO_2$ in air at 37°C. The culture media was replaced every 3-4 days. Cells were passaged at confluence and used within 4 passages. Cultures were inspected using a phase-contrast microscope to confirm typical fibroblast morphology (Fig 2.3).

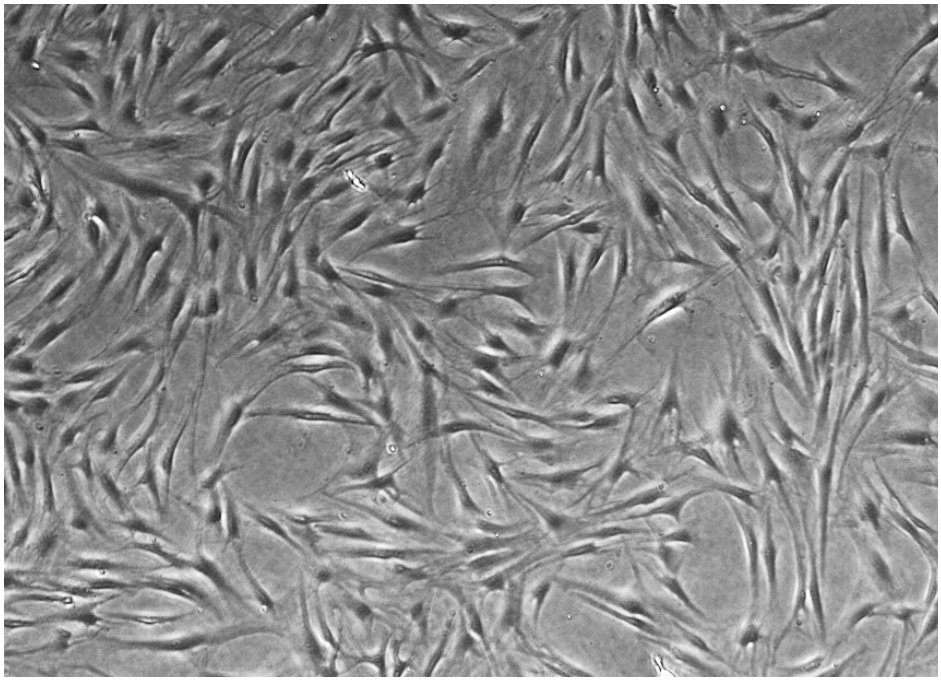

**Figure 2.3. Phase-contrast photomicrographs of primary dermal fibroblast.**

### 2.2.4 Treatments with small molecules

#### 2.2.4.1 TGFβ treatment

In order to clarify the time course of TGFβ1 responsiveness, HaCaT cells were grown in 10% DMEM containing 10% FBS on 6 well plates. At approximately 80% confluence cells were washed twice in PBS, and serum-starved with 0.5% bovine serum albumin for 24 hours, prior to agonist stimulation with recombinant TGF-β1 (R&D) at a concentration of 2ng/ml and 4ng/ml. Cells were monitored for change in morphology and phase-contrast photomicrographs were taken daily. Time points were taken at 0, 0.5, 1, 4, 6, 24, 48 and 72 hours, after which cell monolayers were lysed for mRNA, to assess expression of Snail1 and Snail2.

The immunofluorescent staining and Western blotting assays were performed with HaCaT cells stimulated with 2ng/ml of TGFβ1 for 72 hours.

#### 2.2.4.2 LPS treatment

HaCaT cells were cultured as previously described in 6 well and 12 well plates. At 80 % confluence cells were washed twice in PBS, and serum-starved with 0.5% FBS for 24 hours, prior to treatment with 2ng/ml of LPS. Cells were lysed at 6hr for RNA and at 48hr for proteins.

#### 2.2.4.3 S100A9 treatment

HaCaT cells and primary dermal fibroblasts (passage 3-4) were grown in DMEM supplemented with 10% FBS in 12-well and 96-well plates or chamber slides. At 80%-90% of confluence media was removed, cells washed in PBS and replaced with DMEM containing 0.5% FBS and cultured for further 18-24hrs. Then media was removed and replaced with DMEM supplemented with 0.5% FBS and rhS100A9 (Sino Biological) at concentrations of 2μg/ml, 1.5μg/ml, 1μg/ml and 0.5μg/ml in volumes: 1ml/well of a 12 well plate or per chamber and 120μl/well of a 96 well plate. Cells were maintained for a further 6-48 hours dependent on assay, and visually assessed for viability at 24 and 48hrs.

#### 2.2.4.4 TLR4 inhibitor treatment

Primary dermal fibroblasts (passage 4, SSc n=3, control n=3,) were grown in DMEM supplemented with 10% FBS and P/S in 12-well plates. At around 80% of confluence cells were washed in PBS and cultured in 0.5% FBS P/S DMEM further 24hrs. Next fibroblasts were pre-treated for 1hr with 1μM of selective TLR4 inhibitor TAK-242 (Calbiochem) (Matsunaga et al., 2011) prior to treatment with 1.5μg/ml of rhS100A9 (Sino Biological) in 1ml/well of DMEM 0.5% FBS P/S. Cells were maintained for a further 6hr then lysed for RNA.

### 2.2.5 Cell culture assays

#### 2.2.5.1 MTS assay

Human dermal fibroblasts P4 from 3 healthy controls and 3 SSc patients were seeded in 96 well plate at concentration of 5 000 cells/well and incubated in 10% serum DMEM.

After 24 hours the cells were washed 3x in PBS and switched to 1% serum containing rhS100A9 at concentrations 2µg/ml, 1µg/ml and 0.5µg/ml, TGFβ. The proliferation rate was assessed after 24hrs using CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega). The cells were incubated with 20µl of the solution for 1.5hrs in the in a humidified atmosphere of 5% $CO_2$ in air at 37°C.

#### 2.2.5.2 Crystal violet assay

The effect of rhS100A9, TGFβ and LPS on proliferation of HaCaT cells and primary dermal fibroblasts was assessed by the crystal violet method (Karakecili et al., 2008). The incorporation of crystal violet dye into DNA allows for the sensitive estimation of cell numbers. Upon solubilisation with acetic acid, the amount of dye taken up by the cell monolayer is quantified by optical density (OD) and cell numbers estimated based upon OD at 570nM.

In short, 10 000 cells/well were seeded on 96 well plates in 100µl of DMEM supplemented with 10% FBS and incubated at 37°C, 5% $CO_2$. At 80% confluence cells were serum starved overnight. Then cells were treated with rhS100A9, TGFβ and LPS and 10% FCS was used as a positive control. After 48hrs of treatment cells were washed twice in PBS and excess liquid was carefully removed. Cells were incubated for 10 min at room temperature with 30ul of crystal violet solution (0.1mg of crystal violet, 10ml methanol, 40ml distilled water) per well. Plate was washed thoroughly in tap water and drained upside down on a paper towel to remove the water completely. Then 100ul/well of 33% acetic acid was added and the absorbance was read at 570nm on a plate reader.

#### 2.2.5.3 Viability assay

Human dermal fibroblasts (P4) from 3 healthy controls and 3 SSc patients were seeded in 96 well plate at concentration of 8 000 cells/well and incubated in 10% serum DMEM till

80% confluent. Then the cells were washed 3x in PBS and switched to 1% serum. After 24 hours, proliferation was stopped using mitomycin C (10µg/ml) and cells incubated with serial dilution of rhS100A9 at concentrations 2µg/ml, 1µg/ml and 0.5µg/ml. The effect of rhS100A9 on cells viability was assessed after 72 hours using MTS assay.

#### 2.2.5.4 Scratch assay

Human dermal fibroblasts (P4) from 3 healthy controls and 3 SSc patients were seeded in 96 well plate at concentration of 8 000 cells/well and incubated in 10% serum DMEM till 80% confluent. Then the cells were washed 3x in PBS and switched to 1% serum. After 24 hours, proliferation was stopped using mitomycin C (10µg/ml) and cells incubated with serial dilution of rhS100A9 at concentrations 2µg/ml, 1µg/ml and 0.5µg/ml. The scratch was created and assessed at 24, 48 and 72 hours.

## 2.3 Molecular biology techniques

### 2.3.1 Tissue lysis for RNA

Once equilibrated to room temperature blister epithelial sheets were transferred from RNA-later into a rounded bottom 2ml tubes containing 5mm metal bid, 500µl of RTL buffer and β-mercaptoethanol. The sheets were disrupted in a Tissue Lyser II (Qiagen) for 2 min at 20Hz/s performed twice. Lysates where then transferred to a fresh tube and spun for 3 min at 3000 x g. Supernatants were then used for the RNA extraction.

### 2.3.2 Lysing and homogenisation of cell monolayers for RNA

Media from monolayers of cells was removed and cells washed twice in PBS. Then 400µl of RTL buffer was added to each well and lysate collected using rubber policeman. Lysates were transferred to the 1.5ml Eppendorf tubes and homogenised by passing 5 times through a 20-gauge needle.

### 2.3.3 RNA extraction

Total RNA was extracted using RNeasy kit (Qiagen) according to manufactures instructions. In brief 400µl of 70% ethanol was added to each cell lysates and mixed by vortexing. The samples were then transferred to RNeasy spin columns and centrifuged for 15s at 8000g. Flow-through was discarded; 700µl of RW1 buffer was added to the column and again centrifuged for 15s at 8000g. Next, spin columns were washed in 500µl of RPE buffer and centrifuged for 15s at 8000g, followed by another wash in 500µl of RPE buffer and 2min centrifugation at 8000g. The columns were then transferred to a fresh collection tubes and centrifuged at 1000g for 1min. RNA was eluted in 30µl of RNA/DNA free water.

### 2.3.4 RNA quantification and quality control

RNA was quantified using a Nanodrop ND-8000 spectrophotometer (Thermo-Scientific). Briefly, 1.2µl of samples were placed on the spectrophotometer and measured for quantity and purity estimated using 260/280 and 260/230 ratios. The minimum 260/280 ratio accepted was 1.90 and minimum 260/230 ratio was 1.8. RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent) using the Agilent RNA 6000 Nano kit and chips according to manufacturer's instructions. The RNA integrity algorithm was used to determine integrity. For PCR purposes, minimum acceptable RIN values were >8.0.

### 2.3.5 Reverse transcription

Total RNA was reversely transcribed using QuantiTect Reverse Transcription kit (Qiagen) following standard protocol. In short, genomic DNA was removed by adding 2µl of gDNA wipeout buffer to 1µg of RNA and incubated for 2 min at 42˚C. Then to each sample 1µl of reverse transcriptase and RT Primer Mix containing random and oligoDT primers was added, along with 5µl of RT buffer, and mixed. Samples were incubated for 15 min at 42˚C, followed by 3 min incubation at 95˚C. The cDNA template was then diluted 1 in 10 and stored at -80˚C.

### 2.3.6 Primer design

Primers were designed using NCBI Primer-Blast software (www.ncbi.nlm.nih.gov/tools/primer-blast) and human accession numbers. Intron-spanning amplicons placed towards the 3' end of the gene of interest were preferred. Sequences were analysed for GC content and GC clamps and self-annealing using Vector NTI software. GC content of 40-60, and a difference in Tm $\leq$ 2°C were considered acceptable. Ideal primer length was 18-30 bases and amplicon length 80-200 bp. In order to ensure specificity, primer sequences were subjected to BLAST analysis (www.ncbi.nlm.nih.gov/blast). Specificity was confirmed using melt curve analysis.

### 2.3.7 Assay standards

For some of genes, complementary cDNA standards were amplified from human cDNA known to express the gene of interest. PCR amplification of the amplicon was performed, and the cDNA isolated by gel electrophoresis. Gel purification through the QiaQuick DNA purification columns (Qiagen) was performed. DNA was quantified using the Nanodrop, and these known quantities of amplicon were then diluted to provide eight standards diluted 10-fold in RNAse/DNase free water from $10^7$ copies to $10^1$ copies. These were used to construct standard curves and derive copy numbers and assay efficiencies.

### 2.3.8 qPCR

Diluted cDNA was used in qRT-PCR reactions performed in Rotor-Gene 2000 (Corbett). Each 10µl reaction contained 5µl of SensiMix (Bioline), 0.5µM of both forward and reverse primers, 2µl of nuclease free water and 2µl of cDNA template. The cycling conditions were as follows: initial denaturation at 95°C for 5 min followed by 40 cycles of amplification (denaturation at 95°C for 10s, annealing at 57°C for 20s, and extension for 10 s at 95°C). Samples were run in duplicates and each run included no cDNA

template control. Primers sequences for gene of interest and housekeeping are listed in an Appendix 1.

Target transcript expression was normalised to the expression of the most stable housekeeping gene (*TUBB* for epidermal blisters, *TBP* for fibroblasts and HaCaT cells incubated with S100A9) using previously described the ΔΔCT method (Livak and Schmittgen, 2001). The relative gene expression of a specific gene was calculated according to the equation:

$$\text{Relative gene expression} = 2^{(Ct_{REF} - Ct_{GOI})},$$

Where Ct (cycle threshold) is a number of cycles required for the fluorescent signal to cross the threshold of background fluorescence signal; REF- most stable housekeeping gene; GOI is a gene of interest.

To ensure accuracy of the ΔΔCT method serial dilutions of cDNA were amplified using a range of primers and the efficiency of primers was assessed for each GOI and REF gene. Only primers with efficiency 98% - 100% were used. Normalisation factors for qPCR assays presented in the chapter investigating EMT in SSc skin were calculated for each gene using GeNorm analysis software based on most stable housekeeping gene out of five (Vandesompele et al., 2002). *TUBB* and *B2M* were found to be most stable genes in blister sheets and therefore used for the analysis of *SNAI1* and *SNAI2* in the epidermal sheets; while *TBP* and *B2M* was used in HaCaT cells stimulated with TGFβ.

## 2.4 Immunological assays

### 2.4.1 Measurement of secreted proteins

In order to profile growth factors cytokines and chemokines released by SSc and control epidermis and dermis, media conditioned by the explants was assayed using multiplex platform and ELISAs. Assays sensitivity is listed in the Table 2.3.

**Table 2.3. Luminex and ELISA assays sensitivity.**

| Analyte | Mean assay sensitivity (pg/ml) | Type of assay | Total number of samples |
|---|---|---|---|
| FGF-2 | 0.9 | Luminex | 24 |
| G-CSF | 0.2 | Luminex | 24 |
| GM-CSF | 0.4 | Luminex | 12 |
| IL-1a | 1.1 | Luminex | 24 |
| IL-1b | 0.1 | Luminex | 24 |
| IL-1ra | 0.6 | Luminex | 24 |
| IL-8 | 0.4 | Luminex | 24 |
| MCP-1 | 1.4 | Luminex | 24 |
| VEGF-A | 1.4 | Luminex | 24 |
| PDGF-AA | 0.3 | Luminex | 12 |
| PDGF-BB | 0.7 | Luminex | 12 |
| IL-6 | 1.8 | Luminex | 12 |
| S100A9 | 6.55 | ELISA | 12 |
| HGF | 40 | ELISA/Luminex | 12/12 |
| CCL20 | 0.47 | ELISA | 12 |
| CTGF | 63 | ELISA | 16 |

#### 2.4.1.1 Luminex based multiplex assay

The Luminex assay uses xMAP® technology, which enables simultaneous quantitation of multiple proteins in a sample. Polystyrene color-coded beads, coated with target specific antibodies are mixed together and incubated with the samples. Captured analytes are subsequently detected using biotinylated detection antibodies and a reporter dye streptavidin-phycoerythrin conjugates. In the Luminex analyzer, a red light laser excites the internal dyes that identify each bead, while green light source excites reporter dye bound to the surface of the beads and digital signal processing to effectively allow multiplexing of samples using.

Conditioned media from epidermal and dermal explant experiment were send to Biolegend, where they were analysed for presence of FGF-2, G-CSF, GM-CSF, IL-1a, IL-1b, IL-1ra, IL-8, MCP-1, VEGF-A, PDGF-AA and PDGF-BB, HGF using multiplex platform, Luminex (Biolegend, San Diego, US). Unfortunately, not all assays that I was interested in were available for multiplex analysis at the time. Therefore, samples had to be analysed by separate sandwich ELISAs for the remaining targets: S100A9, CCL20 and CTGF.

#### 2.4.1.2 CCL20 and S100A9 ELISAs

Commercial ELISA kits was used to assess levels of CCL20 (R&D Systems, Minneapolis, US), and S100A9 **(**MBL International, Woburn, US). Standard recommended protocols were followed. In brief, conditioned media from epidermal and dermal explant experiment and appropriate standards was loaded in duplicates on a 96 well plate pre-coated with monoclonal antibodies specific to CCL20/S100A9 and incubated for 2hr/1hr on a plate shaker. Wells were washed and incubated with HRP-conjugated antibody for 2hr/1hr on a plate shaker. Then ELISA plates were washed and TMB substrate solution was added to all wells. The reactions were then stopped using the stop solution (sulphuric acid) once sufficient color development was observed. The absorbance of each well was then analysed by reading the absorbance at wavelengths 450 nm and 540 nm. After subtracting background absorbance, mean value was subjected to

semi-logarithmic analysis and expressed as a concentration in pg/ml. The readings were further analysed in Prism GraphPad.

#### 2.4.1.3 CTGF ELISA

Human CTGF development ELISA kit, which detects free CTGF was purchased from Peprotech. An ELISA protocol provided by the manufacture was followed. In brief, Nunc MaxiSorb plates were coated with 1µg/ml of monoclonal human CTGF coating antibody (100µl per well) over night at room temperature. The plates were then washed 4 times in 0.05% PBST and blocked for 1hr with 300µl per well of blocking solution (sterile filtered 1% BSA in PBS) and washed 4 times with PBST. Next, 100µl of sample/ CTGF standard (serial dilution from 4000pg/ml to 62.5pg/ml) was loaded on a plate and incubated for 2hrs.

Afterwards, plate was washed 4 times and 100µl of biotinylated anti-CTGF detection antibody at concentration of 0.5µg/ml was added. After 2hrs incubation and subsequent series of washing, 100µl of avidin-HRP conjugate was added to wells and incubated for 30min at room temperature. The plate was washed and 100µl of pre-incubated Glo Substrate Reagent A and B mixture (R&D Systems) was added and incubated in the dark for additional 10 minutes. The luminescence of each well was then recorded using 0.1-0.5s readings. After subtracting background luminescence, mean value was subjected to semi-logarithmic analysis and expressed as a concentration in pg/ml. The readings were further analysed in Prism GraphPad.

### 2.4.2 Immunohistochemical analysis of paraffin embedded sections

For formalin-fixed paraffin-embedded (FFPE) specimens, samples were stored overnight in 4% formaldehyde (CellPath, Newtown, UK). Fixed tissues were processed and dehydrated overnight and embedded in molten paraffin wax. 3µm sections were cut on a

Leica UK microtome and mounted onto poly-L-lysine-coated slides (VWR, Lutterworth, UK). After drying at 42°C overnight, sections were stored for staining.

#### 2.4.2.1 Immunohistochemical staining

FFPE sections were de-waxed in xylene (Genta Medical, York, UK) for 10 minutes, rehydrated in gradient concentrations of ethanol and washed in water. Then endogenous peroxidase activity was blocked for 10 min with methanol containing 1% hydrogenpp peroxide. Standard protocols for heat mediated antigen retrieval in citrate buffer (pH 6) or Tris-EDTA (pH 9) for 5-15 minutes were followed in all but pro-collagen staining, where proteinase K digestion was used. Antigen retrieval protocols were optimised for each antibody used.

After cooling down sections were washed in water and unspecific binding was blocked with 10% normal serum (Vector Labs, Peterborough, UK) for 30 minutes. Endogenous biotin activity was then blocked using a commercial avidin/biotin blocking kit (Vector Labs) according to manufacturer's instructions. Diluted in 0.05% PBST antibodies were then applied onto tissue sections and after overnight incubation at 4°C or 1 hour at room temperature sections were washed in PBS. Appropriate IgG controls were included for all immunohistochemical stains. List of primary antibodies used, their dilutions and incubation time was collected in Table2.4.

Optimisation of antibody dilution, length and ambient temperature during treatment was performed for each antibody used. Binding of primary antibodies was detected using an appropriate biotin-conjugated secondary antibody at 1:200 dilution for 30 minutes, followed by 30 min incubation with peroxidise or alkaline-phosphatase substrate (Vector Labs). Sections were developed using 3,3' diaminobenzidine tetrachloride (DAB) as a chromagen or alkaline phosphatase (both from Vector Labs). Sections were counterstained in Mayers' haematoxylin for 30s, dehydrated with alcohol, cleared in xylene, and mounted with a permanent mountant (DPX) (VWR). Where necessary

commercial ImmPress kit was used with rabbit antibodies to aid specificity of staining (Vector Labs).

**Table 2.4. List of primary antibodies used in immunochistochemistry and immunofluorescence.**

| Antibody target | Purchased from | Species | Dilution | Incubation time |
|---|---|---|---|---|
| E-cadherin | BD Biosciences (Oxford, UK) | Mouse monoclonal | 1:100 | Overnight |
| Vimentin | Santa Cruz (Heidelberg, Germany) | Mouse monoclonal | 1:50 | Overnight |
| Cytokeratin 14 (K14) | Vector Labs (Peterborough, UK) | Mouse monoclonal | 1:20 | 1hr |
| Loricrin | Abcam (Cambridge, UK) | Rabbit monoclonal | 1:500 | 1hr |
| Filaggrin | Abcam | Mouse monoclonal | 1:200 | Overnight |
| Involucrin | Abcam | Rabbit polyclonal | 1:500 | 1hr |
| P-smad2/3 | Santa Cruz | Rabbit polyclonal | 1:100 | 1hr |
| FSP-1 | Abcam | Rabbit polyclonal | 1:50 | 1hr |
| Collagen IV | Southern Biotech (Cambridge, UK) | Goat polyclonal | 1:20 | 1hr |
| Ki-67 | Dako (Ely, UK) | Mouse monoclonal | 1:75 | 30 minutes |
| α-SMA | Dako | Mouse monoclonal | 1:75 | 30 minutes |
| S100A9 | Abcam | Rabbit monoclonal | 1:250 | 1hr |
| S100A8 | Abcam | Mouse monoclonal | 1:50 | 1hr |
| Langerin | Abcam | Mouse monoclonal | 1:100 | Overnight |
| Pro-collagen type I | Abcam | Rat monoclonal | 1:800 | 30 minutes |
| Pan-cytokeratin | Santa Cruz | Mouse monoclonal | 1:100 | 1hr |
| HGF | Abcam | Mouse monoclonal | 1:200 | Overnight |
| CTGF | Abcam | Mouse monoclonal | 1:500 | Overnight |
| p-Hspb1 (S78) | Abcam | Mouse monoclonal | 1:500 | Overnight |

Sections were viewed with a Zeiss Axioskop Mot Plus microscope, an Axiocam digital camera using Axiovision software (Zeiss, Cambridge, UK). Figures shown are representative of all sections.

#### 2.4.2.2 Immunofluorescent staining of cells

Cells were grown in 8 well chamber slides (BD Bioscience, Bedford, MA) and after serum starvation treated for 48-72hrs, then washed with PBS and fixed in ice-cold methanol-acetone for 5 min at 4°C. After subsequent PBS wash, unspecific binding was blocked with 10% normal serum (Vector Labs) for 30 minutes. Diluted antibodies to Pan-cytokeratin and FSP-1 (Table 1) were then applied, and after appropriate incubation time cells were washed in 0.05% PBST. Then an suitable fluorophore-conjugated secondary antibody (Life Technologies) at 1:2000 dilution was applied and incubated for 30min in protected from light conditions. Finally, cells were washed and mounted with Vectashield media (Vector Labs) containing 4',6-Diamidino-2-Phenylindole (DAPI) and examined with a Zeiss Axioskop Mot Plus microscope using fluorescence detecting camera (Zeiss).

#### 2.4.2.3 Histological analysis

Sections were viewed with a Zeiss Axioskop Mot Plus microscope, an Axiocam digital camera using Axiovision 4.8 software (Zeiss). Between 6 and 12 sections of both healthy controls and SSc patients were used for the analysis. Microscope measurements, for instance, of epidermal thickness, were performed on 10 high power fields per sample by observers blinded to sample category. Figures shown are representative of all sections.

##### 2.4.2.3.1 Epidermal thickness

In order to determine thickness of the epidermis, an area from the basement membrane to the most inner part of stratum corneum was measured. Mean thickness for each section was calculated and included in statistical analysis. Then from each section an area of 10 cells from basal and spinous layers was measured on 6 fields of view using Axiovision.

The mean was calculated for each section and included in comparison between healthy control and SSc.

#### 2.4.2.3.2 Measurement of loricrin, involucrin and filaggrin bands

For analysis of terminal differentiation skin sections were stained for classical markers: loricrin, involucrin and filaggrin. The expression band thickness for each marker was measured based on 10 measurements taken from each section, and the calculated mean was used to test the difference between healthy and SSc skin.

#### 2.4.2.3.3 Number of proliferating cells

Number of cells positive for proliferation marker – ki67 in the epidermis was counted, averaged for each sample and divided by total number of cells in the basal layer. The result was then expressed as a percentage of the basal cell number and used for analysis.

#### 2.4.2.3.4 Phoshorylated - Smad2/3 in epidermal keratinocytes and papillary dermis

Number of keratinocytes with nuclear phospho-Smad2/3 staining was calculated. Moreover, cells positive for phospho-Smad2/3 in 50μm area in papillary dermis adjacent to basal membrane were examined. The measurements were based on an average from 10 high power fields of view for each section and the numbers compared between controls and SSc in order to assess TGFβ signalling in this area.

#### 2.4.2.3.5 E-cadherin intensity scoring

E-cadherin staining intensity scoring was performed on 6 fields of view for each sample and scored between 1 (the least intense staining) and 3 (the most intense staining). The scores were then averaged for each sample and the mean was used for comparison between control and SSc.

### 2.4.3 Western blot analysis

Western blots of the fibroblast and HaCaT cells were performed to detect levels of E-cadherin, αSMA, FSP-1, type I collagen and CTGF.

#### 2.4.3.1 Preparation of protein cell lysates

Media from dermal fibroblast and HaCaT cells monolayers were aspirated and cells washed three times in PBS. Cells were lysed in 200µl RIPA buffer (Sigma) containing complete protease inhibitor cocktail (Roche, London, UK) and phosphatase inhibitor (Sigma, St. Louis, US). Cells were incubated on ice for 5 min and scraped using rubber policeman. The lysates were then transferred to the Eppendorf tubes and centrifuged at 3000 x g for 10 min to remove cell debris. Total protein was assessed using BCA protein assay (Thermo Fisher, Waltham, US) and aliquots of the lysate were stored at -80°C.

#### 2.4.3.2 Electrophoresis and protein transfer

Samples were prepared by addition of 2µl of a reducing agent and 5µl of a sample buffer (both Life Technologies) to 13µl of cell lysates and heated for 10 min at 70°C. Samples were then loaded on 4-12% Bis-Tris polyacrylamide gels (Life Technologies) at 16µl per well, alongside protein marker (Life Technologies) and electrophoresed in MOPS SDS buffer (Life Technologies) for 50min at 200V. Afterwards, proteins were blotted on nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK) for 1.5hr at 30V and the membranes incubated for 1hr in blocking buffer (5% milk 0.05% PBST) on the rocking platform. The blots were subsequently probed overnight/1hr with primary antibodies diluted in the blocking buffer, followed by washing in 0.05% PBST and 1hr incubation with appropriate biotinylated species-specific secondary antibodies diluted 1:1000. After subsequent washing the blots were visualised using chemiluminescent detection system (GE Healthcare) and developed against photographic film (GE Healthcare). Finally, optical density of bands was calculated and presented in graphs. List of primary antibodies including dilution factors are available in Table 2.5.

**Table 2.5. List of primary antibodies used in Western blotting.**

| Antibody target | Purchased from | Species | Dilution | Incubation time |
|---|---|---|---|---|
| E-cadherin | BD Biosciences | Mouse monoclonal | 1:1000 | Overnight |
| FSP-1 | Abcam | Rabbit polyclonal | 1:750 | Overnight |
| Collagen type I | Millipore (Nottingham, UK) | Goat monoclonal | 1:1000 | Overnight |
| CTGF | Abcam | Mouse monoclonal | 1:500 | Overnight |
| α-SMA | Dako | Mouse monoclonal | 1:1000 | Overnight |
| β-tubulin | Abcam | Rabbit polyclonal | 1:10000 | 2hr |
| GAPDH | Abcam | Goat monoclonal | 1:20000 | 1hr |

### 2.4.4 Phosphorylation microarray

Forearm skin biopsy samples from SSc (n=4) and control (n=4) subjects were immediately frozen in liquid nitrogen in order to prevent the decay of phosphorylation events. Epidermis was separated surgically and samples were shipped to Kinexus Bioinformatics (Vancouver, Canada), where they were lysed and processed using phospho-site specific antibodies. This service uses the KAM-1.2 chip with two samples analyzed at a time utilising 500 pan-specific antibodies (for protein expression) and 300 phospho-site-specific antibodies (for phosphorylation) in duplicate for at least 248 different phospho-sites, 193 protein kinases, 24 protein phosphatises and 150 regulatory subunits of these enzymes and other cell signalling proteins that regulate cell proliferation, stress and apoptosis.

## 2.5 Statistical analysis

For quantitative variables, the mean ± standard deviation or, where appropriate, standard error of the mean from replicate samples, or from independent experiments were calculated. Summary data are presented as mean ±SD/SEM of n observations. Statistical analysis was assessed as indicated in the text with means compared using either the Student's t-test (in vitro experiments with primary fibroblasts and HaCaT cells) included in the Microsoft Excell package (Redmond, US), one-way ANOVA with post hoc analysis using Tukey's multiple comparison test (mRNA analysis of blister sheets), or nonparametric Mann-Whitney U test (proteome and phosphor-smad2/3 analysis) using the GraphPad Prism 5 software (La Jolla, US). A p value $\leq 0.05$ was considered as statistically significant.

Permutation testing for phosphorylation array data was performed using an Excel add-in, Significance Analysis of Microarray Data (Tusher et al., 2001) and followed by calculation of the false discovery rate (Hochberg and Benjamini, 1990).

# Chapter Three

# Results: Morphological Characteristics of SSc Epidermis

## 3.1 Introduction

SSc is a complex rheumatic connective tissue disease with wide spectrum of symptoms. However, skin involvement is one of the most common features and its severity is often used as a marker of disease severity and prognosis. Some of the cardinal features of the SSc skin include fibrosis, thickening, abnormal pigmentation and alopecia. Over the years a lot of efforts have been put into better understanding of processes associated with skin fibrosis, but the vast majority of studies have concentrated on the dermal component of the skin. Therefore, data regarding SSc epidermis and characteristics of its keratinocytes are very limited. Even reports on basic morphological features of the SSc epidermis are sporadic and often inconsistent. For example epidermal thickness in SSc vary between publications, from hypertrophic (Van Praet et al., 2011, Rossi et al., 2010, Sauermann et al., 2002, Aden et al., 2008), through normal (Morley et al., 1985a), to atrophic (Cooper et al., 1979). Likewise, previous reports cannot agree on proliferation status of the keratinocytes in SSc skin, and include hyperplasia (increase in cell number) (Milano et al., 2008), as well as a non- significant increase in number of proliferating cells (Pablos et al., 1999).

Histological studies performed in our lab have shown changes in the SSc epidermis in the pattern of K14 and K1/K10 expression. Moreover, proteomic analysis revealed altered levels of proteins involved in epidermal differentiation, such as increase in caspase 14 precursor, K1 and K14, but decrease in K5, when compared with control (Aden et al., 2008). The presence of nuclei in the SSc stratum corneum (parakeratosis) was also previously reported (Van Praet et al., 2011, Maeda et al., 1993). However, those changes consistent with altered differentiation of SSc keratinocytes were never fully investigated.

Following on from these findings I started systematically analyse the epidermis in SSc. As discussed in the introduction section of this thesis, normal human epidermis comprises of proliferating basal keratinocytes and suprabasal keratinocytes, which stop dividing and undergo terminal differentiation. These two types of keratinocytes can be distinguished by the pattern of cytokeratins, and expression of structural proteins of the cornified-

envelope (Watt et al., 1993, Candi et al., 2005). The mitotically active basal progenitor cells, express K5 and K14 but do not synthesise proteins associated with the cornified-envelope. Upon migration to suprabasal position the expression of cytokeratins switch to K1 and K10, and they start synthesis of structural envelope proteins such as involucrin, loricrin, and filaggrin, which later become cross linked by Ca2+ dependent transglutaminase. During the process, keratinocytes become gradually flattened and loose their intracellular organelles, including nuclei. This journey ends in stratum corneum, from where the cornified envelopes are finally removed from the skin surface.

The differentiation process is closely regulated, so that the number of proliferating and differentiating keratinocytes at any given time is closely coupled, giving the epidermis of the same thickness and mechanical strength. However, this process is altered following injury to the skin. Keratinocytes at the wound edge can withdraw from the terminal differentiation process to enable sufficient number of cells for wound re-epithelialisation. An activated phenotype of keratinocytes around the site of injury is associated with a change in the pattern of cytokeratins expression. Synthesis of normally absent K6, K16 and K17 is induced, while cytokeratin 14 continues to be expressed in suprabasal layers (Usui et al., 2005). These changes are also mirrored in the expression of cornified envelope proteins, with markers of terminally differentiating keratinocytes expressed closer to the basal layer than normally (Li et al., 2000).

Protein phosphorylation is one of the forms of post-translational modifications that affect protein activity. Upon introduction of a phosphate group some proteins become activated while others, become inhibited. Therefore, unsurprisingly reversible phosphorylation of proteins controls almost all aspects of cell life, while abnormal action of protein kinases and phosphatases responsible for phosphorylation and de-phosphorylation disrupts intra- and extracellular processes leading to pathology. Therefore, distinguishing phosphorylation status of proteins in SSc epidermis would allow a better understanding of changes in the diseased epidermis.

The aim of this chapter is to further characterize the SSc epidermal keratinocytes. Differences in morphology such as, epidermal thickness and number of keratinocytes, between normal and SSc epidermis are examined. In addition, comparison of proliferation rate between the SSc epidermis and control epidermis is made, and expression of standard differentiation markers is investigated in cutaneous sections. Furthermore, differences in phosphorylation of epidermal proteins reported by Aden (Aden et al., 2010) are further evaluated.

## 3.2 Results

Figures 3.1 to 3.7 were adapted from the paper (Nikitorowicz-Buniak et al., 2014).

### 3.2.1 Epidermal thickness

Although dermal thickness is increased in SSc, reports on epidermal thickness are not consistent (Morley et al., 1985b, Van Praet et al., 2011, Rossi et al., 2010). Therefore, to assess the extent of epidermal thickness, histological studies were performed on full thickness skin biopsies stained with hematoxylin. This assessment revealed that the epidermis in the majority of SSc sections contained more layers of cells, when compared with control tissue (Fig.3.1a). The mean thickness ± SEM of control epidermis was 51.3μm ± 7.58 and 88.9μm ± 4.20 for SSc (Fig.3.1b). The observed thickening of SSc epidermis was statistically significant ($p<0.05$).

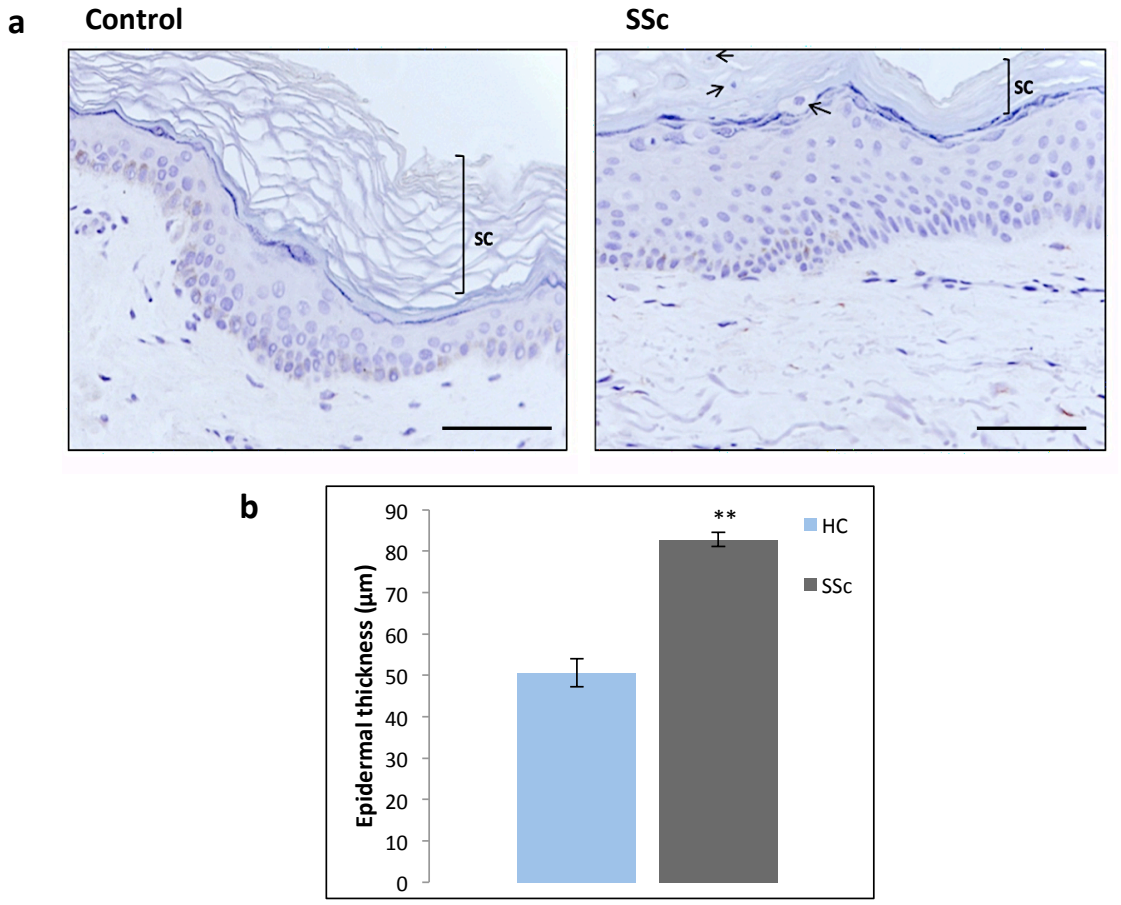


**Figure 3.1 Comparison of epidermal thickness between control and SSc.**

**(a)** Control and SSc skin sections were stained with hematoxylin. Parakeratosis in SSc stratum corneum (SC) was marked using arrows. Representative images chosen to analyse **(b)** thickness of epidermis was measured from the basal to the cornified layer (HC n=6, SSc n=6). For each section 5 images were taken at 20X magnification and 10 measurements were taken from each image to calculate the mean thickness for each section. The results are presented as mean ± SEM. Statistical significance was determined using the Mann-Whitney U test **$p<0.05$.

### 3.2.2 Keratinocyte hypertrophy

To find out whether the thickened epidermis contained more and/or larger cells, the cells were counted (Fig.3.2a) The mean cell number 307.1±71.37 observed in SSc epidermis was significantly higher when compared to healthy subjects 209.6±47.81 ($p<0.005$). Moreover, during counting of keratinocytes, it has been noticed that the cells in the SSc epidermis seemed to be larger. The link between the size of epidermal cells and its differentiation status has been established in 70s' (Sun and Green, 1976). As discussed in the introduction chapter of this thesis, bigger cell size is characteristic to differentiating cells. As the cell increases in size, it loses ability to divide, but starts expressing differentiation markers, such as involucrin. Thus, to investigate if the SSc keratinocytes are indeed larger than those in control epidermis, the area of basal (dividing) and spinous (differentiating) cells was measured. Basal cells of healthy controls had mean area of 74.76μm ±8.03, while SSc were significantly bigger 111.71μm ±16.71 ($p<0.05$) (Fig.3.2b). Cell area of spinous layer keratinocytes (Fig.3.2c) was also significantly increased, with 91.97μm ±14.17 in healthy epidermis and 165.09μm ±43.90 in SSc ($p<0.05$).

Since the epidermal layer has significantly more keratinocytes, the proliferation rate may be enhanced in SSc. However, the hypertrophy of SSc keratinocytes would point to higher number of cells that are undergoing terminal differentiation. Therefore both of those processes were investigated in more depth.

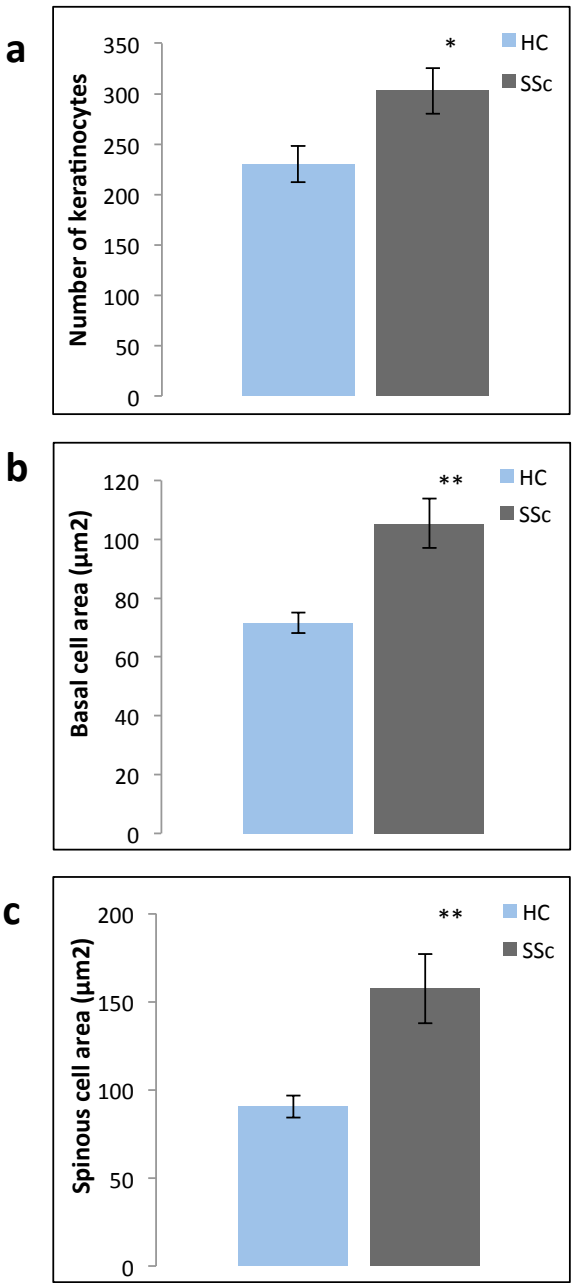


**Figure 3.2 Activated keratinocytes in SSc epidermis.**

**(a)** The mean number of epidermal keratinocytes was established based on 6 images at 20X magnification for each section (HC n=5, SSc n=6). The area of 10 cells in basal and spinous layers was measured at 5 separate 20X images for each section (HC n=5, SSc n=6) and the mean area of the cells in **(b)** basal and **(c)** spinous epidermal layers was calculated for SSc and control epidermis. The results are presented as mean ± SEM. Statistical significance was determined using the Mann-Whitney U test *$p<0.05$, ** $p<0.005$.

### 3.2.3 Keratinocytes proliferation

In order to determine, if the increase in cell number in SSc epidermis is due to over proliferation, normal and SSc sections were stained with a proliferation marker, ki-67 (Fig.3.3a). Positively stained cells in both normal and SSc epidermis were located within 1-3 layers from the basal membrane. The mean percentage of keratinocytes expressing ki-67 in the SSc epidermis was 8.4±2.19 and healthy subjects 3.3±3.66 (Fig.3.3b). Although, the difference between control and SSc was relatively large, the variation between the samples meant that only a trend towards increased (p=0.235) was observed.

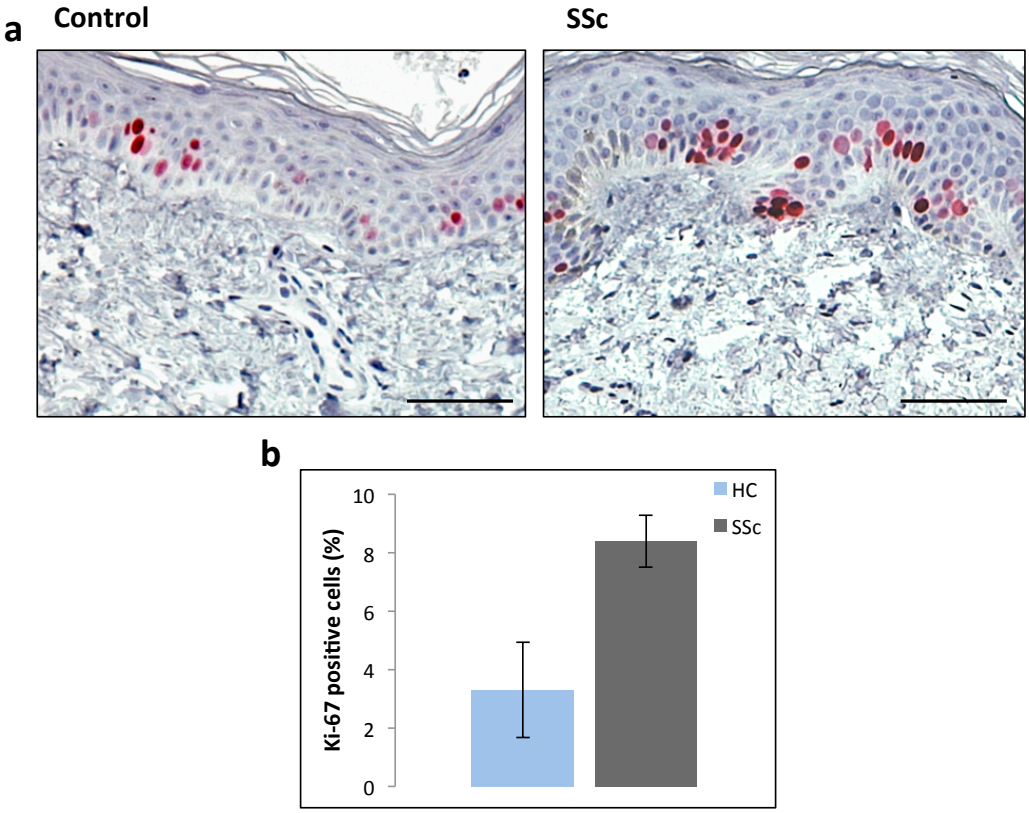


**Figure 3.3 Keratinocytes proliferation in SSc epidermis.**

**(a)** The expression of proliferating cells in the epidermis of SSc patients and controls was detected using anti-ki-67 antibody. **(b)** The mean percentage of proliferating cells was calculated from an average of 6 measurements of ki67 positive cells taken from each section, divided by total number of cells in the basal layer (HC n=5, SSc n=6). The results are presented as mean ± SEM. Statistical significance was determined using the Mann-Whitney U test. P value was non-significant.

### 3.2.4 Keratinocytes terminal differentiation

As shown in Figure 3.1a, in some SSc sections nucleated cells were still present within stratum corneum, indicating parakeratosis. Moreover, the cornified layers of these biopsies had tightly packed corneocytes whereas, in healthy epidermis corneocytes were spaced, and individual ones could be recognised. The findings were consistent across early and established dcSSc, and suggest that terminal differentiation may be either delayed or incomplete. Furthermore, the hypertrophy of SSc keratinocytes indicates that the differentiation process in the SSc epidermis is altered. Also as already mentioned, reports from our lab showed altered expression of marker of basal keratinocytes K14, that is not confined to basal layer in SSc, but also expressed in suprabasal layers, suggesting altered differentiation in SSc keratinocytes. Therefore, to assess if expression of other markers of keratinocyte differentiation process was also changed in SSc, sections were stained with antibodies against classical terminal differentiation markers, involucrin, loricrin and filaggrin.

The cells positive for involucrin in control samples were present only in the granular layer of the epidermis (Fig.3.4). However, in SSc epidermis the staining was extended downwards to the spinous layer keratinocytes, with all but basal cells positive for involucrin.

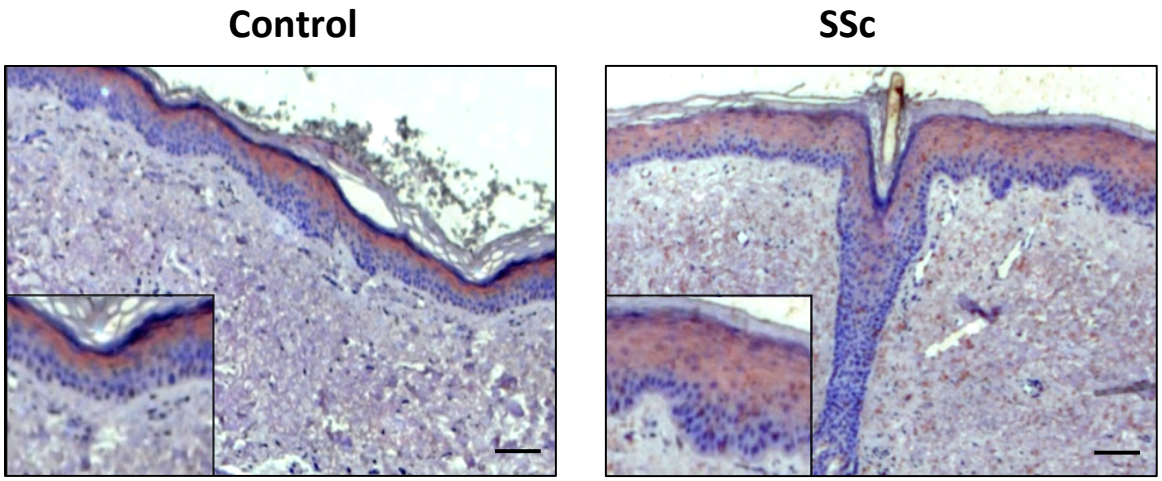


**Figure 3.4 Abnormal expression of involucrin in SSc epidermis.**

The expression of differentiation marker involucrin in the epidermis of SSc patients and controls was detected by immunohistochemical staining using anti-involucrin antibody. Sections were counterstained with hematoxylin.

Similar results were observed with loricrin, which stained positive just 1 layer below stratum corneum in controls, but in SSc epidermis strong staining was also seen few layers down, and weak staining in all but basal keratinocytes (Fig.3.5).

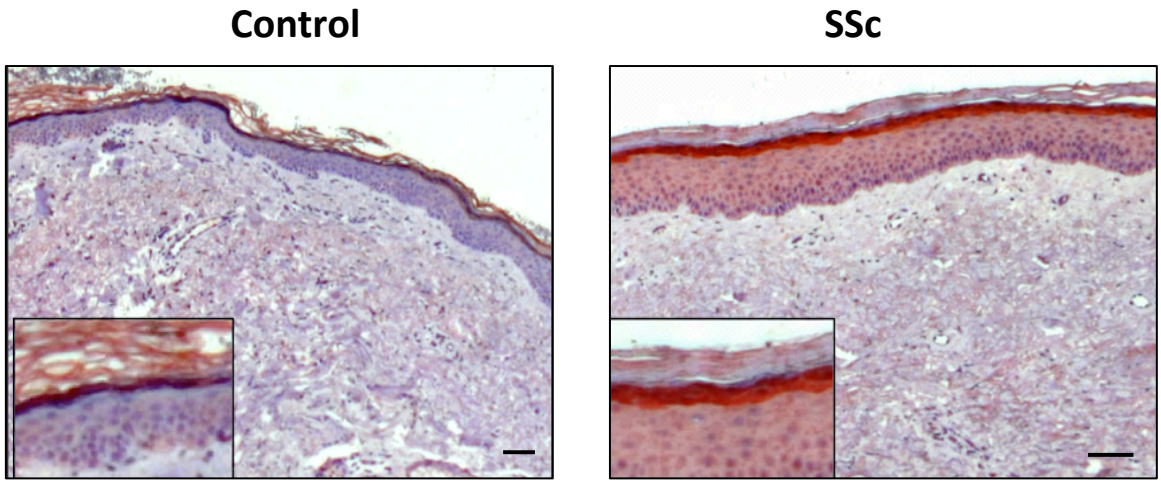


**Figure 3.5 Abnormal expression of loricrin in SSc epidermis.**

The expression of differentiation marker loricrin in the epidermis of SSc patients and controls was detected by immunohistochemical staining using anti-loricrin antibody. Sections were counterstained with hematoxylin.

Finally, filaggrin in controls stained single layer of cells below stratum corneum, but in SSc the staining was observed in more layers of cells (Fig.3.6). Moreover, loricrin along with involucrin and filaggrin seem to appear in relatively lower epidermal layers during differentiation of SSc epidermal keratinocytes, closer to the basal layer. One possible explanation is that although, cells enter a differentiation process, they travel towards the stratum corneum at a slower rate and the process of their removal from cell surface is delayed in SSc.

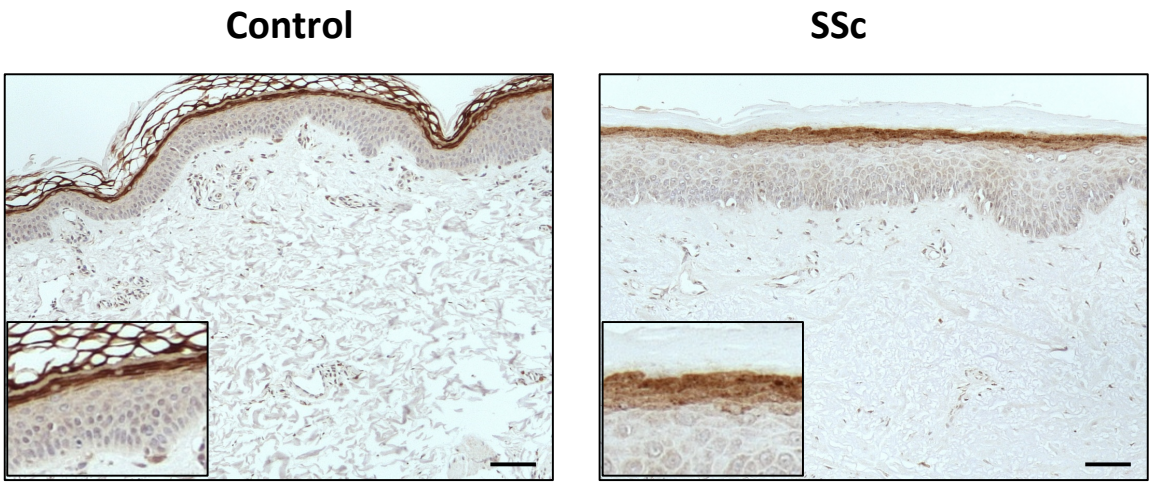


**Figure 3.6 Abnormal expression of filaggrin in SSc epidermis.**

The expression of differentiation marker filaggrin in the epidermis of SSc patients and controls was detected by immunohistochemical staining using anti-filaggrin antibody. Sections were counterstained with hematoxylin.

Moreover, when quantified, the mean depth of involucrin, loricrin, and filaggrin staining was increased over 2 fold in SSc epidermis compared to controls (Fig.3.7). The mean depth of involucrin staining in SSc epidermis was 22.5µm ±22.85 compared to 15.15µm ±0.97 in controls ($p<0.05$). Similarly, loricrin depth in SSc was 8.80µm ±7.21 compared to 4.83µm ±1.20 in controls ($p=0.05$), and filaggrin depth in SSc was 6.03µm ±1.72 and 2.24µm ±0.22 in controls ($p<0.0005$). The expansion in number of layers expressing terminal differentiation markers indicates that the cornification process is delayed. This could partially explain increase in epidermal thickness seen in SSc. Additionally, such abnormal differentiation and increased cell number could impact on signalling and

protein secretion from the epidermis and therefore alter epidermal-dermal cross talk.

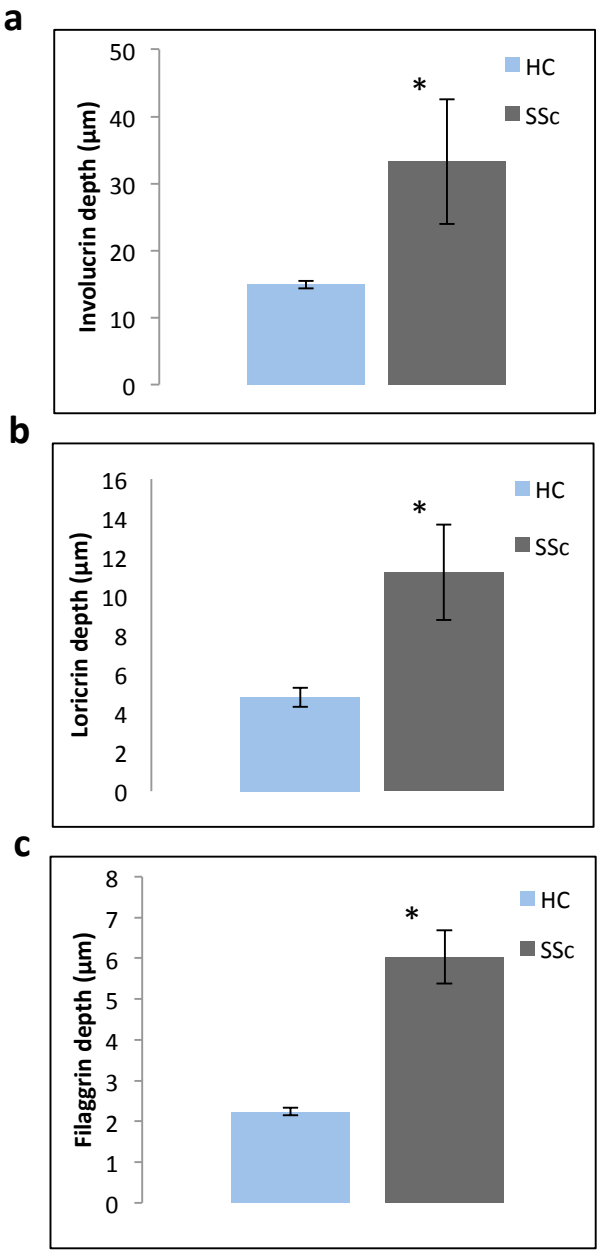


**Figure 3.7 Quantitative analysis of involucrin, loricrin and filaggrin staining.**

Representative images such as the one in the previous figures 3.4 - 3.6 were chosen to analyse expression bands of **(a)** involucrin (HC n=5 SSc n=7) **(b)** loricrin (HC n=6 SSc n=9) and **(c)** thickness of filaggrin (HC n=5 SSc n=7). The mean thickness for each band was calculated from 10 measurements taken from each section. The results are presented as mean ± SEM. Statistical significance was determined using the Mann-Whitney U test *$p<0.05$.

Heat shock protein 27 (HSBP1), a member of small heat shock protein is also involved in keratinocytes differentiation (Hell-Pourmojib et al., 2002), and its expression in normal keratinocytes correlates with differentiation status. HSBP1 undergoes phosphorylation and redistribution to the cytoskeleton during the late phase of epidermal keratinocyte differentiation (Robitaille et al., 2010, Jonak et al., 2011, Gandour-Edwards et al., 1994).

The abnormal expression pattern of HSBP1 in SSc epidermis was earlier reported (Aden et al., 2008), with the protein stained not only suprabasal but also basal layers of keratinocytes. However, the phosphorylation status of HSBP1 in the SSc epidermis was never previously investigated. As demonstrated in this chapter the differentiation process of SSc keratinocytes is altered and delayed. Therefore, I anticipated differential expression of phosphorylated HSBP1 between control and SSc sections. Indeed the staining revealed changes to the location of the protein between control, early and late SSc (Fig.3.8).

As expected control sections showed strong cytoplasm staining of phospho-HSBP1 in the granular layer. However, sections of early SSc showed also staining in the lower suprabasal layers of the epidermis. Although basal cells of control and early SSc epidermis were negative for phospho-HSBP1, the late disease sections staining was less heterogeneous with positive cells also in the basal layer. Further demonstrating differences in keratinocyte differentiation associated proteins between control and SSc epidermis, as well as changes with disease progression.

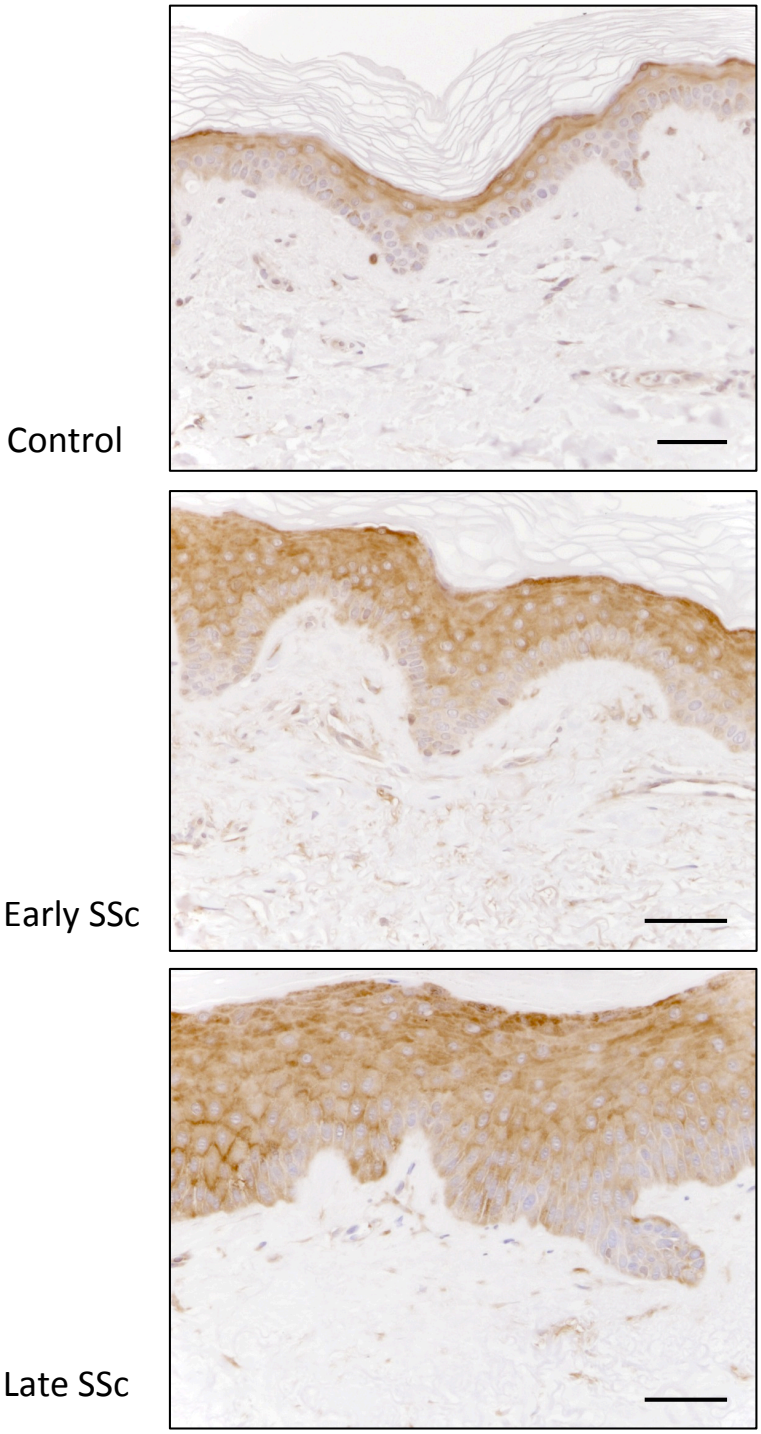


**Figure 3.8 Enhanced phosphorylation of heat shock protein 27 (HSBP1) in SSc epidermis.**

The expression of phosphorylated HSBP1 in the epidermis of SSc patients and controls was detected by immunohistochemistry. Sections were counterstained with hematoxylin.

### 3.2.5 Phosphorylation of proteins in SSc epidermis

Kinex™ antibody microarray was previously performed on epidermal tissue in my department to detect of phosphorylation of more than 240 protein kinases, 28 phosphatases, and 90 other cell signalling proteins implicated in cell proliferation, stress and apoptosis (Aden et al., 2010). The results showed an altered pattern of phosphorylation in SSc epidermis. Among those differentially phosphorylated were proteins involved in signal transduction pathways, cell cycling and cell metabolism participants. However, only proteins with 2 fold difference and above were included in the analysis. The remaining data still contains proteins with significantly altered expression and phosphorylation. As small difference in phosphorylation level may have a large impact on cellular processes I decided to perform analysis of interactions between all proteins with significantly upregulated or downregulated phosphorylation in SSc. In order to better understand processes important in disease pathogenesis I investigated using String software (von Mering et al., 2003, von Mering et al., 2005, Jensen et al., 2009, Franceschini et al., 2013) in which biological processes these proteins are involved.

#### 3.2.5.1 Proteins with upregulated phosphorylation or aboundance in SSc epidermis detected by Kinexus

Kinexus assay results revealed 20 proteins with increased phosphorylation levels in SSc compared with controls. Some proteins were phosphorylated at just one site others at a few. These proteins amongst others included phosphatases and kinases, as well as proteins with well-established links with SSc, such as Smad2 and PDGF receptor. The whole list of proteins with increased phosphorylation along with their phosphorylation sites, and fold difference is presented in a table 3.1.

**Table 3.1. Increased proteins phosphorylation in SSc epidermis.**

| Protein | Phospho site | Fold increase | p-value |
|---|---|---|---|
| 40S ribosomal protein S6 (RPS6) | S235 | 1.47 | 0.0072 |
| p38 alpha (MAPK14) | T180+Y182 | 1.20 | 0.0086 |
| Protein kinase D (PRKD1) | S738+S742 | 1.63 | 0.0092 |
| p70/p85 ribosomal protein-serine S6 kinase alpha (RPS6KB1) | T229 and S221/S227 | 1.52 and 1.43 | 0.0109 and 0.0319 |
| Platelet-derived growth factor receptor kinase alpha (PDGFRA) | Y754 and Y572+Y574/ Y579+Y581 | 1.75 and 1.63 | 0.0174 and 0.0363 |
| Protein-serine kinase C gamma (PRKCG) | T655 and T514 | 1.22 and 1.86 | 0.0182 and 0.0394 |
| Tyrosine hydroxylase isoform a (TK) | S18 | 1.94 | 0.0218 |
| Integrin alpha 4 (INTGR4) | S988 | 1.15 | 0.0224 |
| Microtubule-associated protein tau (MAPT) | S518 | 1.42 | 0.0241 |
| Phosphoprotein-enriched in diabetes/astrocytes 15 (PEA15) | S116 | 1.58 | 0.0256 |
| SMA- and mothers against decapentaplegic homolog 2 (Smad2) | S465+S467 | 1.64 | 0.0293 |
| p70/p85 ribosomal protein-serine S6 kinase alpha (RPS6KA1) | T389 | 1.35 | 0.0294 |
| Protein-serine kinase C delta (PRKCD) | S645 | 1.53 | 0.0321 |
| N-methyl-D-aspartate glutamate receptor 1 subunit zeta (GRIN1) | S896 | 1.19 | 0.0335 |
| G protein-coupled receptor-serine kinase 2 (GRK2) | S670 | 1.27 | 0.0389 |
| MAPK/ERK protein-serine kinase 1 (MAP2K1) | T291 | 1.48 | 0.0389 |
| Synapsin 1 isoform Ia (SYN1) | S9 | 1.32 | 0.0398 |
| Protein-serine kinase C beta (PRKCB) | T641 | 1.30 | 0.0467 |
| Myosin regulatory light chain 2, smooth muscle isoform (MYLK2) | S20 | 1.33 | 0.0473 |
| Protein-tyrosine phosphatase 1D (PTPN11) | S576 | 1.83 | 0.0478 |

To better visualize the interactions between those proteins String analysis was performed (Fig.3.9)

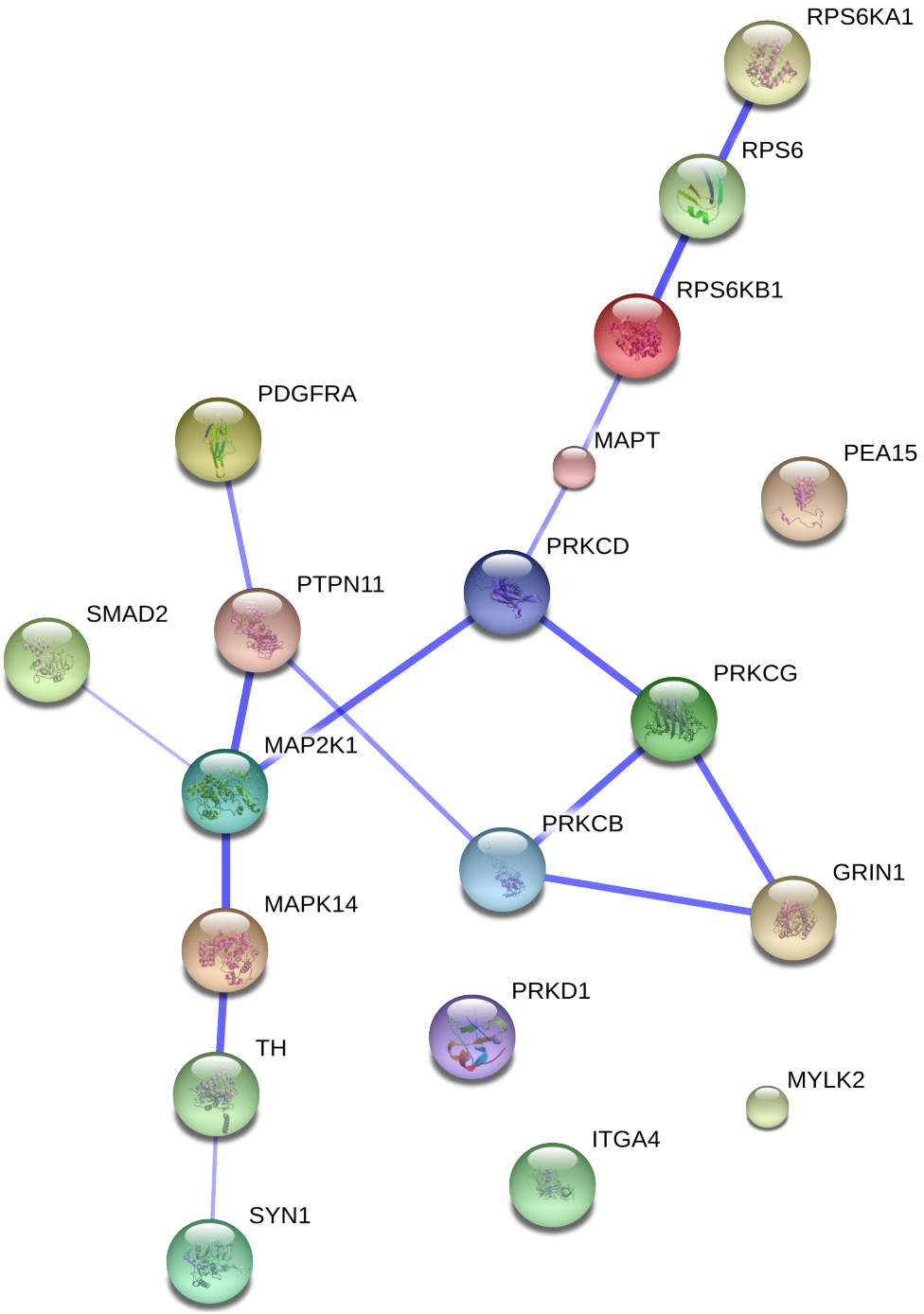


**Figure 3.9 Model of predicted interactions between proteins with significantly higher phosphorylation status in SSc epidermis.**

Interactions between proteins with increased phosphorylation in SSc epidermis biopsies compared to controls (as identified by phospho-protein microarray assay performed on control epidermis n=4 and SSc epidermis n=4) were modeled using String 9.1 software. The lines between nodes represent protein associations, with stronger associations represented by thicker lines.

According to algorithms used by the String software majority of the proteins with higher phosphorylation interact together; however PEA15, INTGR4, PRKD1 and MYLK2 did not seem to link with other proteins from the list. Then the biological processes involving the proteins with upregulated phosphorylation were identified using enrichment GO Biological Processes available within the software. The processes after adjustment for false discovery rate included: response to growth factor (9 proteins), cell surface receptor signaling (13 proteins), protein phosphorylation (8 proteins), response to wounding (9 proteins), blood coagulation (7 proteins), hemostasis (7 proteins), positive regulation of biosynthetic process (9 proteins), platelet activation (5 proteins), regulation of response to stress (7 proteins), cell death (8 proteins), innate immune response (6 proteins), cell-cell signaling (7 proteins), and positive regulation of cell migration (4 proteins). The wide range of different pathways in which the upregulated proteins take part, is mainly due to the fact that most of those proteins are enzymes with multiple substrates.

Some of the proteins had not only increased phosphorylation, but were also more abundant, namely PTPN11, MAPK14, PPP1CB, PRKCG, RPS6, RPS6KB1. According to PhosphoSite database (www.phosphsite.org) PTPN11, PPP1CB and calcium-activated PRKCG are involved in cell motility, polarity and chemotaxis. However, there is no data available on the phosphorylation of the particular sites indicated by Kinexus assay, similar with RPS6KB. RPS6 participates in regulation of molecular association. MAPK14 phosphorylation at T180 and Y182 induces its enzymatic activity and is implicated in altered apoptosis, cell adhesion, cell cycle regulation, cell motility, as well as cytoskeletal reorganization. Alterations in those activities are possibly able to contribute to fibrosis and inflammatory changes seen in SSc.

In order to gain a more comprehensive view of the protein interactions in the SSc epidermis, significantly increased proteins detected with Pan-specific antibodies of the Kinexus array were also included in the analysis. The model of interactions between the 52 proteins of SSc epidermis, generated by String software showed interlinked pathways involved in many different biological processes (Fig.3.10). Again most of the proteins formed a complex network of interconnected interactions.

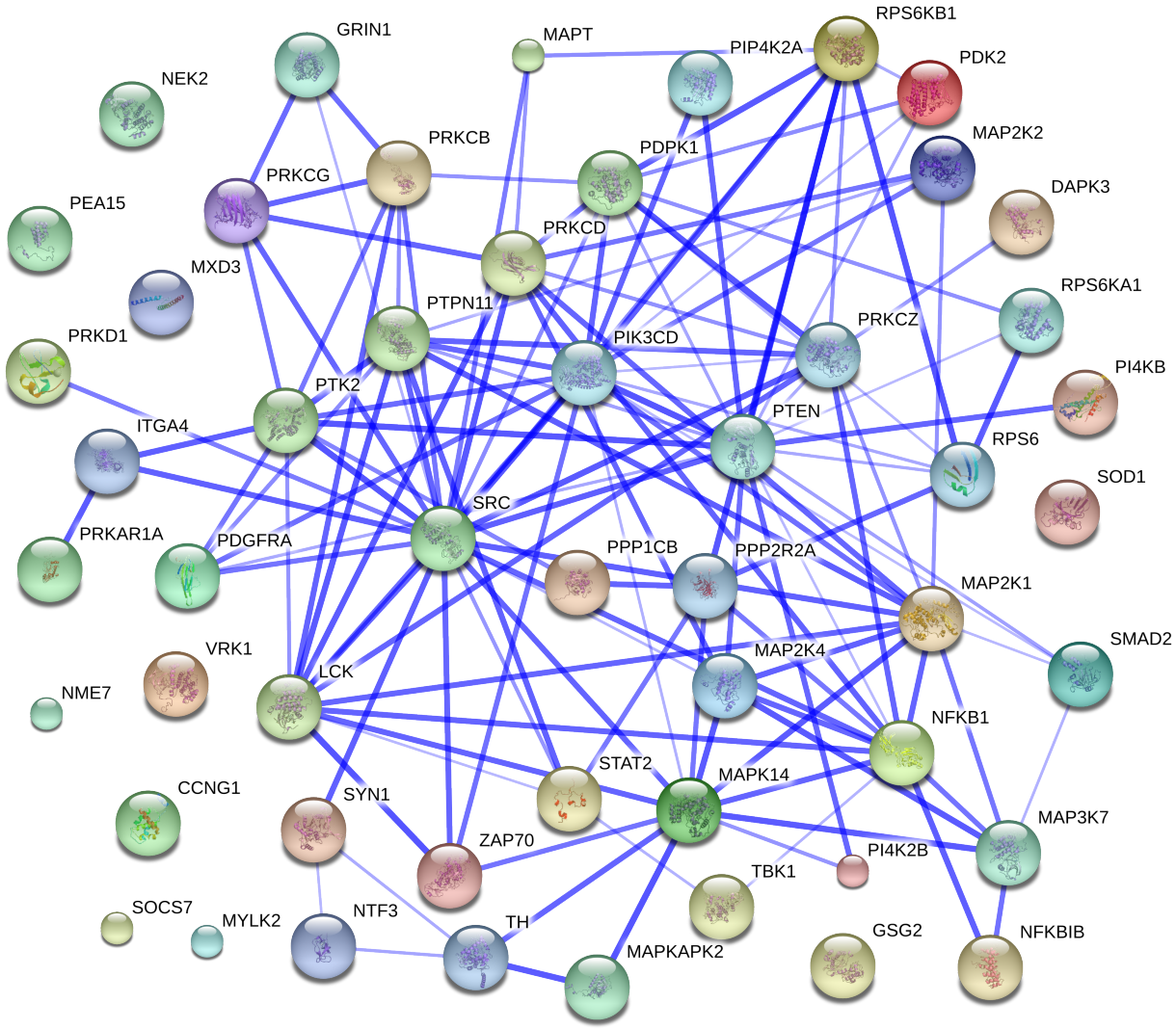


**Figure 3.10 Model of interactions between proteins with significantly abundance and higher phosphorylation status in SSc epidermis.**

Interactions between proteins with increased phosphorylation and abundance in SSc epidermis biopsies compared to controls were modeled using String 9.1 software. The lines between nodes represent protein associations.

Then proteins were subdivided by involvement in biological processes and their p values adjusted for false discovery rate. As presented in table 3.2, results indicate that SSc epidermal cells have phenotype similar to that seen during wound healing, and have activated response to stress. Additionally, when compared to controls, the SSc epidermis has increased activation of innate and adaptive immune system, signal transduction, pathways implicated in platelet activation and blood coagulation.

**Table 3.2. Biological processes with increased proteins phosphorylation and/or abundance in SSc epidermis.**

| Biological process | p-value | Number of proteins |
|---|---|---|
| Protein phosphorylation | 2.24e-16 | 23 |
| Immune response-activating signal transduction | 7.10e-11 | 13 |
| TRIF-dependent toll-like receptor signaling | 3.86e-10 | 9 |
| Response to growth factors | 4.04e-10 | 13 |
| TLR4 signalling | 1.41e-9 | 9 |
| Intracellular signal transduction | 1.44e-9 | 22 |
| Cell surface receptor signaling | 3.06e-9 | 26 |
| Pattern recognition receptor signaling | 8.66e-9 | 9 |
| Innate immune response activation | 1.09e-8 | 9 |
| Response to wounding | 9.04e-8 | 18 |
| Platelet activation | 2. 94e-7 | 10 |
| Blood coagulation | 4.91e-7 | 13 |
| Hemostasis | 5.07e-7 | 13 |
| Regulation of response to stress | 2.29e-6 | 14 |
| Cell activation | 7.58e-6 | 12 |
| Fibroblast growth factor receptor signaling | 1.55e-5 | 7 |
| Epidermal growth factor receptor signaling | 3.69e-5 | 7 |
| Negative regulation of cell death | 3.69e-6 | 12 |
| T cell receptor signaling | 6.69e-5 | 6 |
| Regulation of cell differentiation | 8.74e-4 | 12 |
| Leukocyte migration | 2.98e-3 | 6 |
| Signal transduction | 3.23e-3 | 23 |
| Cellular response to stimulus | 3.38e-3 | 26 |
| Cell-cell signalling | 4.02e-3 | 11 |
| Posttranscriptional regulation of gene expression | 4.54e-3 | 7 |
| Regulation of gene expression | 6.97e-3 | 21 |
| Regulation of cell mobility | 1.08e-2 | 7 |
| Chemotaxis | 1.23e-2 | 8 |

p value after correction for false discovery rate.

#### 3.2.5.2 Proteins with downregulated phosphorylation levels or abundance in SSc epidermis

Kinexus had also identified 17 proteins with decreased phosphorylation levels in SSc epidermis compared to control. The table 3.3 shows the details, including phosphorylation site and fold decrease. Interestingly, MAPT phosphorylation in SSc epidermis was reduced at one site while increased at the other when compared to control. Similar was true for phosphorylated HSBP1, for which immunostaining of skin sections revealed increased abundance and in the phospho-array was decreased, however the antibodies used in these techniques were designed to bind to different phosphorylation sites. Unfortunately, PhosphoSite database does not contain details on the effects of phosphorylation of particular locations for MAPT and HSBP1. MAPKAPK2 phosphorylation activates its enzymatic activity, and HSP27 is one of its substrates. The induction of MAPKAPK2 signaling was shown to be a response to cytokines, stress and chemotactic factors, which is consistent with the hypothesis of activated epidermis in SSc.

Increased phosphorylation at S78 is responsible for cell cycle regulation as is phosphorylation of H3.3 and INSR at that specific site according to PhosphoSite database. This along with previous results in this chapter showing abnormal differentiation of keratinocytes further indicates that SSc epidermal keratinocytes have an altered cell cycle. PTK phosphorylation at these particular sites activates the enzyme responsible for: cell adhesion, differentiation, stimulation of cell motility, cytoskeletal reorganization and transcription regulation. Cytoskeletal reorganization is also linked to phosphorylation of ADD1. CAV2 actions induced by phosphorylation include signal transduction, control of cellular growth control and apoptosis. While SAPK phosphorylation induces apoptosis and alters cell adhesion and transcription. Binding of phosphor into CTNNB1 at S45 leads to alteration of cell adhesion and motility, as well as protein degradation. In contrast phosphorylation of B23 at T199 inhibits its activity.

While phosphorylation of FOXO3 at position T32 also inhibits its activity, as well as alters genes transcription.

**Table 3.3. Decreased proteins phosphorylation in SSc epidermis.**

| Protein | Phospho site | Fold decrease | p-value |
|---|---|---|---|
| Heat shock 27 kDa protein beta 1 (HSPB1) | S15 | 0.64 | 0.0007 |
| Acetyl coenzyme A carboxylase (ACACB) | S80 | 0.53 | 0.0008 |
| Histone (H3.3) | S11 | 0.63 | 0.0016 |
| Lymphocyte-specific protein-tyrosine kinase (LCK) | S157 | 0.72 | 0.0164 |
| Mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2) | T222 | 0.76 | 0.0178 |
| Focal adhesion protein-tyrosine kinase (PTK2) | Y576 and S722 | 0.77 and 0.57 | 0.0226 and 0.0466 |
| Adducin alpha/gamma (ADD1/3)* | S726/S693 | 0.78 | 0.0235 |
| Microtubule-associated protein tau (MAPT) | S530 | 0.53 | 0.0270 |
| Nucleophosmin (B23) | T199 | 0.61 | 0.0337 |
| Stress-activated protein kinase 1/2/3 (MAPK8/11/12) | T183+Y185 | 0.53 | 0.0354 |
| Caveolin 2 (CAV2) | S36 | 0.44 | 0.0447 |
| Insulin receptor (INSR) | Y999 | 0.57 | 0.0448 |
| Catenin (cadherin-associated protein) beta 1 (CTNNB1) | S45 | 0.51 | 0.0474 |
| Forkhead-like transcription factor 1 (FOXO3) | T32 | 0.80 | 0.0502 |

p value after correction for false discovery rate; * not included in the String analysis

When modeled the interactions using String software majority showed close connections (Fig.3.11), similar to the results of upregulated phosphorylation. Those interactions indicated involvement of these proteins into several biological processes, including cell signaling and phosphorylation, as well as responses to growth factors.

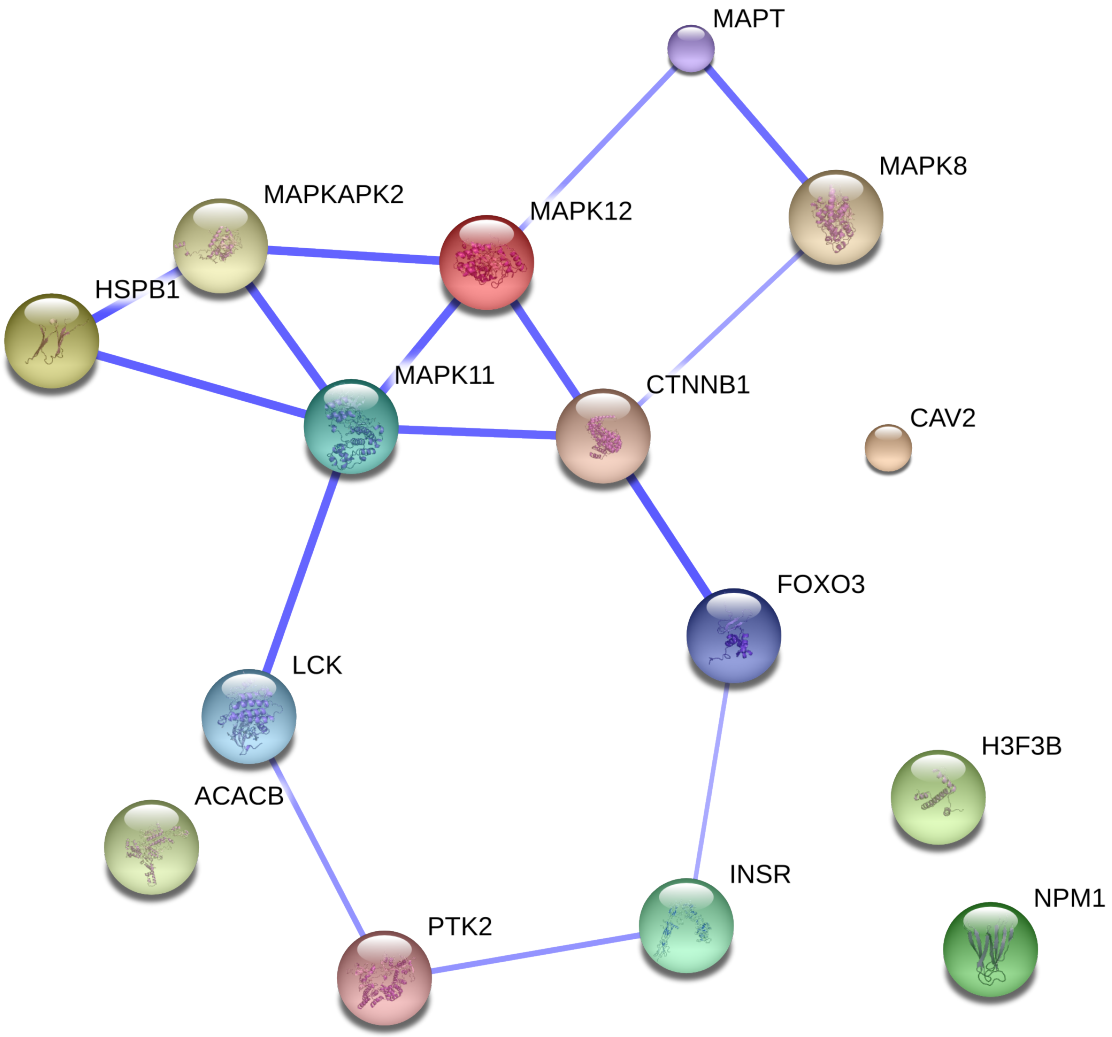


**Figure 3.11 Model of predicted interactions between proteins with significantly lower phosphorylation status in SSc epidermis.**

Interactions between proteins with decreased phosphorylation in SSc epidermis biopsies compared to controls (as identified by phosphoprotein microarray assay performed on control epidermis n=4 and SSc epidermis n=4) were modeled using String 9.1 software.

The model of interactions between 40 proteins, which showed decreased phosphorylation levels or reduced expression in SSc epidermis (Pan-specific results), was then generated by String software. Its outcome showed interlinked pathways involved in many different biological processes (Fig.3.12).

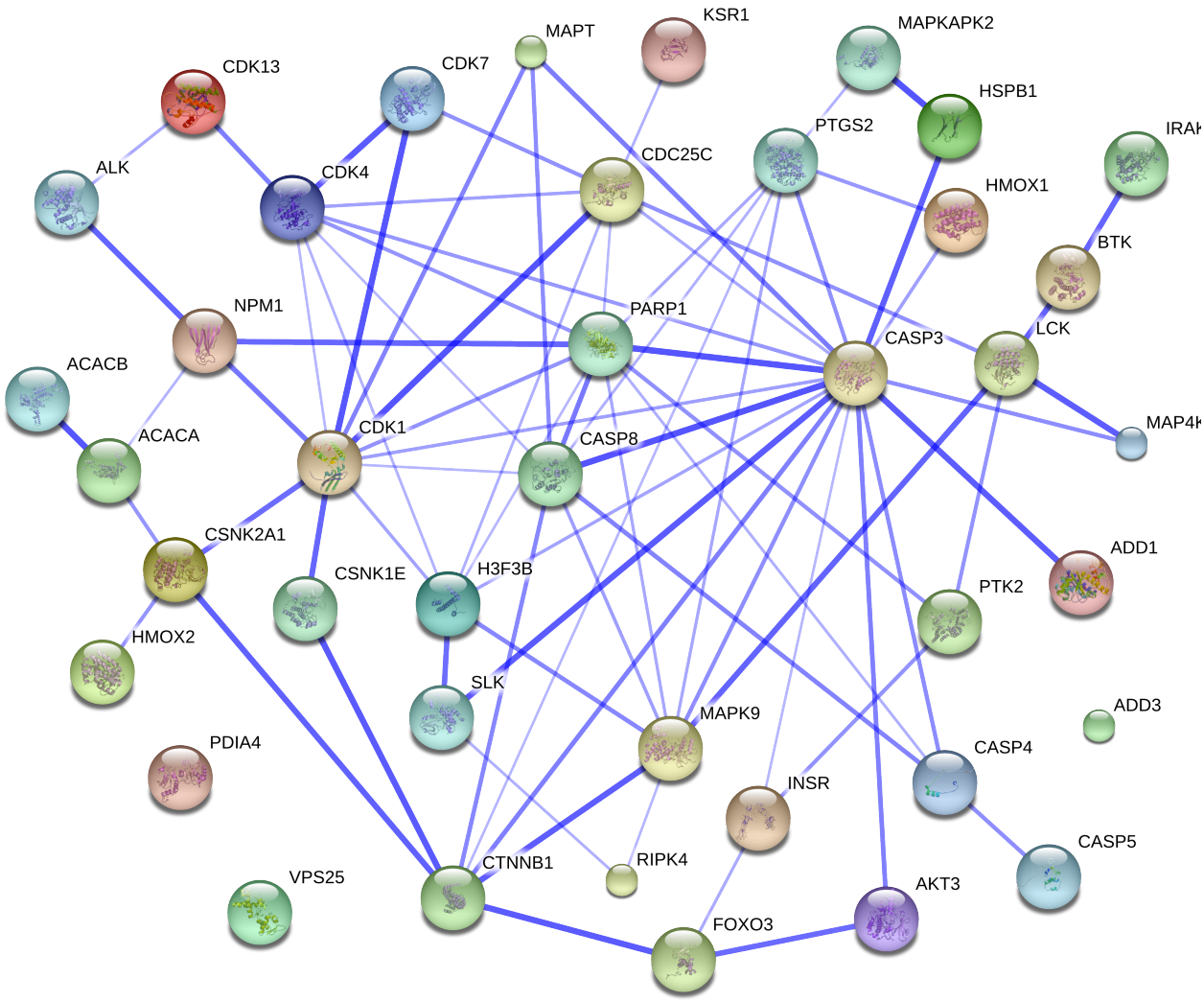


**Figure 3.12 Model of predicted interactions between proteins with significantly lower phosphorylation status or reduced abundance in SSc epidermis.**

Interactions between proteins with decreased phosphorylation in SSc epidermis biopsies compared to controls (as identified by phosphoprotein microarray assay performed on control epidermis n=4 and SSc epidermis n=4) were modeled using String 9.1 software. The lines between nodes represent protein associations, with stronger associations represented by thicker lines.

The proteins characterized by reduced phosphorylation in SSc epidermis were then analysed using the String enrichment option for biological processes with false discovery rate correction. Those processes that reached significant p-value had a wide range and besides protein phosphorylation included, response to stress, DNA damage and growth

factors, as well as cell cycle regulation, immune response, and protein biosynthesis (Table 3.4). Although, proteins present associated with wound healing and keratinocyte differentiation were also identified amongst these with reduced abundance in SSc, the overall change in phosphorylation of proteins from these processes did not reach significance.

**Table 3.4. Biological processes with decreased proteins phosphorylation or abundance in SSc epidermis.**

| Biological process | p-value | Number of proteins |
|---|---|---|
| Protein phosphorylation | 7.029e-7 | 14 |
| Protein kinase activity | 0.0002 | 11 |
| Response to stress | 0.0018 | 12 |
| Regulation of protein metabolism | 0.0013 | 13 |
| Cellular protein modification | 0.0012 | 16 |
| Pattern recognition receptor signalling | 0.0019 | 5 |
| Activation of innate immune response | 0.0019 | 5 |
| Regulation of cell cycle | 0.0024 | 10 |
| Response to DNA damage | 0.00604 | 8 |
| Response to growth factors | 0.0064 | 6 |
| Regulation of defense response | 0.0073 | 7 |
| TLR4 signalling pathway | 0.0076 | 4 |
| Positive regulation of apoptosis | 0.01 | 8 |
| MAPK cascade | 0.016 | 5 |
| Protein complex biogenesis and assembly | 0.016 | 8 |
| Cell death | 0.023 | 10 |
| Response to hormone stimulus | 0.032 | 8 |
| Positive regulation of cell proliferation | 0.052 | 7 |
| Homeostasis | 0.053 | 9 |

p value after correction for false discovery rate.

The figure 3.13 illustrates that many of the SSc epidermal proteins with lower phosphorylation are implicated in more than one biological function.

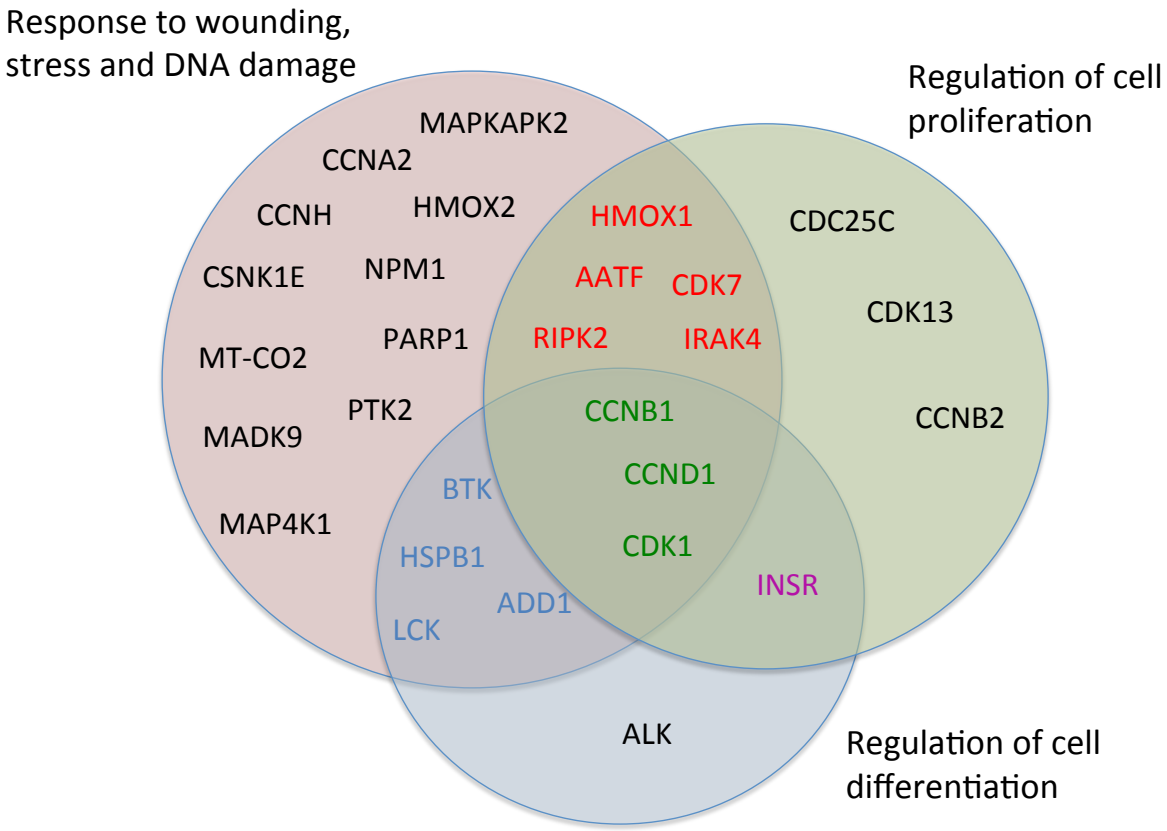


**Figure 3.13 Clautering of proteins with decreased phosphorylation in SSc epidermis into major groups controling cellular proliferation, differentiation and response to wounding and stress.**

Proteins with decreased phosphorylation in SSc epidermis biopsies compared to controls and were analysed for biological functions using String 9.1 software. The schematic representation demonstrates proteins belonging to three main identified clusters relevant to disease process and function of the epidermis.

In order to determine, if the SSc keratinocytes phenotype characterized by the changes described above will remain present during cell culture, I have tried to establish primary keratinocyte cultures. However, despite all the efforts I did not managed to optimize the protocol (see Appendix 2) to get enough cells from patients and controls to carry out these experiments. The main reason seems to be a limited number of actively proliferating keratinocytes that was isolated from 4mm forearm biopsy. The cells attached to the matrix produced by pre-seeded 3T3 fibroblasts divided slowly and most of the time started to differentiate before reaching a level of confluence required to passage or cryopreserve (Fig 3.14). Even though, some strains grew better after passaging them for just one time, they either did not attach to the dish or quickly started to differentiate.

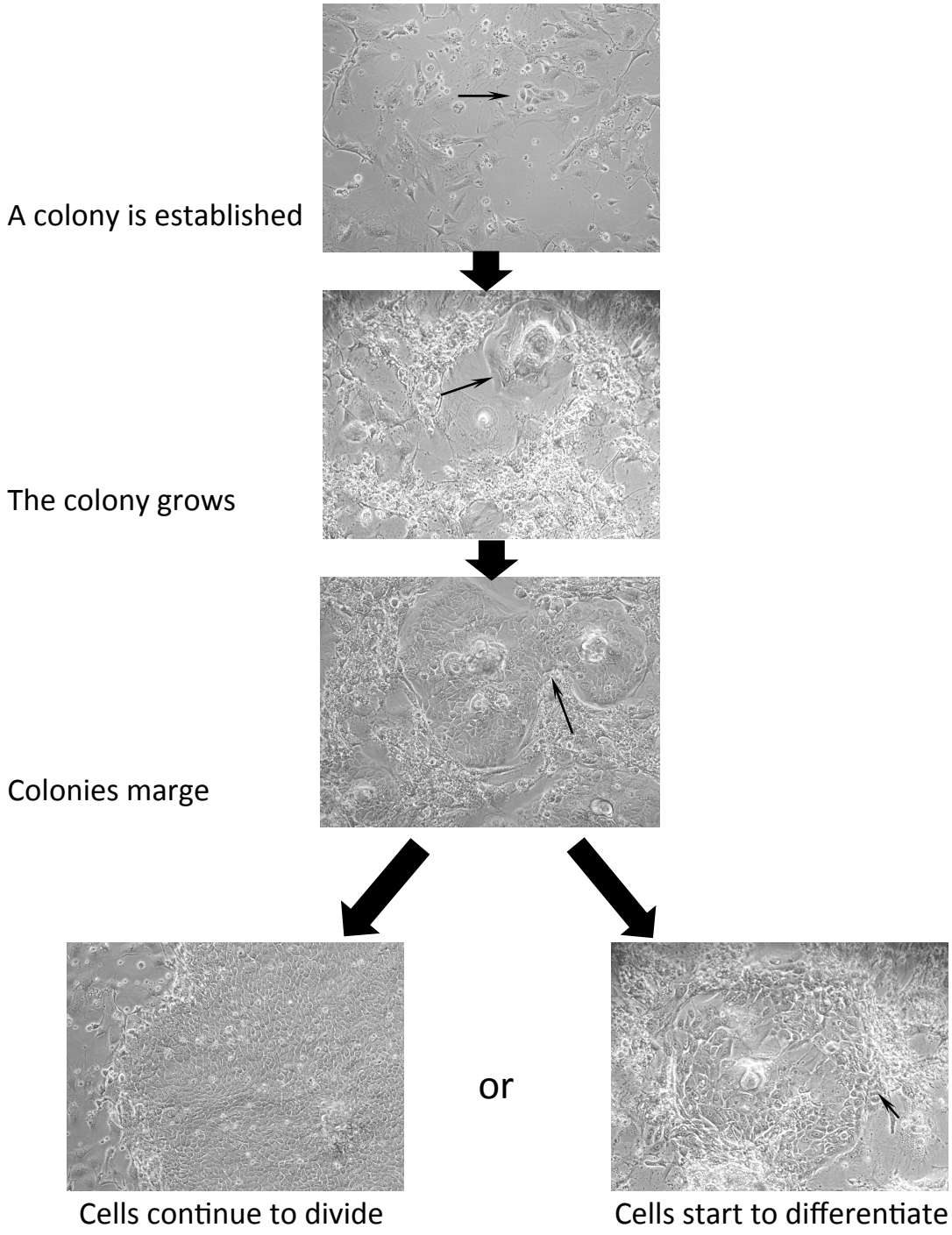


**Figure 3.14 Culture of primary human keratinocytes using 3T3 feeder cells.**

Images of keratinocytes were taken during different stages of cell culture.

## 3.3 Discussion

In this chapter analysis of morphological characteristics of SSc epidermis was performed, in order to increase the limited existing knowledge of this part of the skin in the disease. The main emphasis was on comparing phosphorylation of proteins, and expression levels of proteins involved in differentiation and proliferation between control and SSc epidermis. Due to large differences between diffuse and limited SSc subsets, and to avoid additional variability between samples, and simplify interpretation of the results in my work I have focused on the dcSSc subset. As demonstrated above, SSc epidermis differs from control epidermis in many important aspects of morphology, protein expression and phosphorylation.

The observed significant increase in epidermal thickness between basal layer and stratum corneum confirms some of the previous reports (Van Praet et al., 2011, Rossi et al., 2010). This points to unbalance between epithelial cell proliferation and rate of terminal differentiation, which in normal conditions contributes to the overall epidermal thickness. In the healthy epidermis, proliferation at the basal layer is precisely balanced with desquamation in the cornified layer. Given the increased thickness in SSc skin, I sought to assess these two processes to determine whether increased proliferation, altered differentiation, or a combination of both was occurring.

The analysis of the stratification process revealed presence of nuclei in the SSc stratum corneum (parakeratosis) in some of the SSc sections. This was consistent with previous reports (Van Praet et al., 2011, Maeda et al., 1993). Additionally, in SSc epidermis key structural cornified-envelope proteins: involucrin, loricrin and filaggrin were found much closer to the basal layer of keratinocytes than in healthy subjects, where the expression was limited to top granular layers. This indicates similarities between SSc epidermis and epidermis during wound re-epithelialisation (Mansbridge and Knapp, 1987). Such premature expression of differentiation markers was also reported to be associated with hyperproliferative keratinocytes of wounds and psoriasis skin (Hertle et al., 1992, Li et al., 2000). Moreover, the significantly thicker bands of expression of those cornified

envelope precursors, along with parakaretosis and atypical tightly packed stratum corneum, further points to an abnormal terminal differentiation process in SSc skin. Such partial differentiation could provide the basis to the thickening of epidermis seen in the disease.

Another contributor to the SSc epidermal hypertrophy could be the observed increase in number of keratinocytes with a trend towards increase in proliferation marker ki-67. Previous reports concerning SSc keratinocytes proliferation were inconsistent and included hyperplasia (Milano et al., 2008), non- significant increase in number of proliferating cells (Pablos et al., 1999) as well as epidermal atrophy (Cooper et al., 1979). There may be several reasons of discrepancies between these results, including differences in patients cohorts of these studies, such as disease duration, mRSS, age of participants, and subset of SSc patients used in the study (Milano et al., 2008).

The significant hypertrophy of basal and spinous keratinocytes may also be a factor contributing to the increased thickness of SSc epidermis. Keratinocyte size is known to be linked to its differentiation status; smaller size is associated with DNA synthesis and inhibition of differentiation, while larger size with stimulation of differentiation and inhibition of cell division (Watt and Green, 1981). The presented evidence is consistent with other results of keratinocyte differentiation analysis in SSc epidermis, described in this chapter. Thus further strengthen evidence of disrupted terminal differentiation of SSc keratinocytes, and supports previous report of abnormal expression of K14 and K1/K10 in SSc epidermis (Aden et al., 2008).

Heat shock protein 27 (HSBP1) is a member of small heat shock protein family, which acts as molecular chaperone able to sequester damaged proteins and prevent their aggregation. This property is regulated via reversible phosphorylation in response to variety of stimuli including mitogens, and inflammatory cytokines such as IL-1β and TNFα. The phosphorylation of HSBP1 by mitogen-activated protein kinase-activated kinases 2 and 3 (MK2/3) or PCK delta is Akt or ERK-dependent (Gandour-Edwards et al., 1994, Maizels et al., 1998). Phosphorylation of HSBP1 results in dissociation of large

multi-meric complex and loss of chaperoning activity (Rogalla et al., 1999). Other functions of HSBP1 include control of apoptosis (Arrigo, 2000), differentiation (Hell-Pourmojib et al., 2002) and cell migration through regulation of actin remodeling.

The non-phosphorylated form of HSBP1 inhibits actin polymerization while its phosphorylation promotes actin polymerization and cell migration (Benndorf et al., 1994). In addition to its role in actin remodeling, HSBP1 contributes to the expression of pro-inflammatory mediators including IL-6 and IL-8 (Alford et al., 2007). Moreover, HSBP1 expression and phosphorylation was demonstrated to regulate fibroblast adhesion, and wound contraction (Hirano et al., 2004, Hirano et al., 2002). HSBP1-deficient mice show a significant impairment in wound healing associated with collagen deposition, increased inflammation and enhanced neutrophil infiltration (Crowe et al., 2013). Therefore, discovery of increased phosphorylation at S82, with simultaneous reduction of phosphorylation at S15 in the SSc epidermis might be important. However, due to the limited knowledge as to the effect of these phospho sites on the HSBP1 functions, it is difficult to discuss the implications beyond keratinocyte differentiation.

Unfortunately, the observations of altered differentiation and proliferation of SSc keratinocytes could not be modeled *in vitro* due to issues with primary keratinocytes cell culture. However, the experiments with primary keratinocytes and fibroblasts are essential to confirm the effect of SSc epidermis on dermal fibroblasts (see chapter 4), thus it is a key issue to establish successful cell culture of this cells. To achieve this aim a bigger size of biopsy could be attempted or else optimally growing strains of SSc keratinocytes could be immortalized. The last option is not ideal due to the fact that a strain with enhanced proliferation is selected, which does not have to represent the majority of keratinocytes. Moreover, during the immortalization process itself some changes could be introduced, that could lead to further differences between immortal and typical SSc keratinocytes. Nevertheless, overall this could be outweighed by benefits of almost unlimited number of SSc keratinocytes.

All the results presented in this chapter indicate abnormal differentiation of SSc keratinocytes, which could be an effect of contact with stiff and fibrotic ECM of the SSc dermis, or differential expression of soluble factors by different skin components. Such alteration in keratinocytes differentiation combined with abnormal phosphorylation could be linked to cell activation, and impact on cell signalling and protein secretion form the epidermis, and therefore modify epidermal-dermal cross talk, as well as interactions with other cell types. Alterations of protein phosphorylation affecting a variety of different biological processes suggest, that they may be associated in SSc skin pathogenesis, either by generating pathological changes or as a compensatory mechanism to those changes. Thus highlighting the idea that not one single pathway could be responsible for SSc skin disease, but many are involved in creation of the SSc phenotype.

# Chapter Four

# Results: SSc Epidermis Contribution to Fibrosis and Inflammation

## 4.1 Introduction

SSc is a rheumatic connective tissue disorder of unknown etiology, and its common features include autoimmunity, inflammation, and vasculopathy, leading to fibrosis (Kahaleh and LeRoy, 1999). Central to SSc pathogenesis is fibroblast activation (Jimenez and Bashey, 1977, Abraham et al., 2007), which in SSc lesions persist as myofibroblast, with enhanced ability to produce collagens, adhere, and contract extracellular matrix (ECM) (Jimenez et al., 1986). Although, the majority of studies were performed on the dermis, there is increasing evidence supporting a key role for the epidermis in promoting dermal fibrosis (Bellemare et al., 2005, Simon et al., 2011, Niessen et al., 2001, Meyer et al., 2011, Funayama et al., 2003, Mustoe and Gurjala, 2011).

Epithelial cells during tissue repair can stimulate fibroblasts via interleukin 1 (IL-1) (Maas-Szabowski and Fusenig, 1996) to secrete growth factors and cytokines essential for epithelial cells growth (Smola et al., 1993). The keratinocytes can also regulate fibroblast expression of ECM modulating genes such as CTGF and type I collagen (Koskela et al., 2010, Ghaffari et al., 2009). In organotypic co-cultures with epithelial cells, fibroblasts contract more readily than in monolayers (Shephard et al., 2004a). The correct epithelial-mesenchymal interactions are essential for skin homeostasis (Szabowski et al., 2000, Maas-Szabowski et al., 2000) and repair (Werner et al., 2007, El Ghalbzouri and Ponec, 2004). These interactions are based on the production of soluble factors (Smola et al., 1993, Denton et al., 2009, Ghahary et al., 2004) and cell-matrix interactions (Gailit and Clark, 1994, Watt and Fujiwara, 2011).

A previous report from our lab has demonstrated that SSc keratinocytes are activated and resemble those seen in wound healing (Aden et al., 2008). Injured epidermal cells are known to secrete pro-inflammatory cytokines and chemokines (Luger and Schwarz, 1990), as well as growth factors affecting the phenotype and proliferation of dermal fibroblasts (Werner et al., 2007). Considering abnormal differentiation and increased cell number in SSc, which were presented in the previous results chapter of this thesis, it is possible that such anomalies can be associated with changes in soluble factors released by

epidermis and dermis.

In order to establish if that is the case I have used profiling of key growth factors and cytokines implicated in SSc pathogeneis and in keratinocyte-fibroblast cross talk, including IL-6, IL-8, CCL20, MCP-1 and S100A9 which were used as markers of inflammation, as well as IL-1α, IL-β, and their inhibitor IL1-ra. In keeping with this idea I selected targets which were previously reported to be elevated in the skin of SSc patients, including inmunostaining showing increased expression of IL-6 (Khan et al., 2012), CCL20 (Tao et al., 2011), and MCP-1 (Karrer et al., 2005) by SSc keratinocytes and fibroblasts. Also during the timecourse of the work on this thesis data showing increased S100A9 in SSc was reported (Xu et al., 2013b). However, there is no information on SSc epidermal expression of other targets, including IL-1α as researchers have focused on dermal part of the skin (Kawaguchi 2004*).

CTGF and PDGF were used as fibrotic markers, while HGF as anti-fibrotic marker implicated in epithelial repair. All of these were previously reported to be increased in the SSc skin (Igarashi et al., 1996, Frost et al., 2012, Yamakage et al., 1992), but without specific analysis of their role in the disease epidermis. VEGF was used as indicator of angiogenesis and it has been previously described to be elevated in SSc keratinocytes (Davies et al., 2006). FGF-2, a major growth factor released by keratinocytes to stimulate growth of fibroblasts also known to be overexpressed in the SSc skin (Lawrence et al., 2006) was use as indicator of epidermal- dermal cross talk.

This chapter focuses on assessment of differences in concentrations of these key soluble factors. Their release by epidermal and dermal explants into conditioned media was measured using Luminex or ELISA, and the results were validated by immunohistochemistry analysis of skin sections, as well as evaluation of mRNA levels in the epidermal blister sheets.

## 4.2 Specific methods

### 4.2.1 Lactate dehydrogenase (LDH) assay

Aliquots of 50µl of the media conditioned overnight with SSc or control dermis, and epidermis were transferred to 96-well flat bottom plate in duplicate wells and assayed using LDH Cytotoxicity Assay Kit (Thermo Scientific). In brief to each sample 50µl of the Reaction Mixture was added and mixed. The plate was then incubated at room temperature for 30 minutes protected from light. After that time, the Stop Solution was added and mixed, and absorbance was measured at 490nm and 680nm on microplate reader (Mithras LB940). After correcting for blank sample absorbance (serum free media without cells), cytotoxicity was calculated for each sample by subtracting the absorbance at 490nm from the absorbance at 680nm.

## 4.3 Results

Figures 4.1 to 4.8, 4.10, 4.13 to 4.17, 4.20 and 4.21 were adapted from the paper (Nikitorowicz-Buniak et al., 2014).

### 4.3.1 Secrotome assay analysis

#### 4.3.1.1 LDH assay

LDH is an enzyme present in the cytoplasm, often used in cell viability and cytotoxicity assays. Upon damage to plasma membrane, the membrane integrity is lost and LDH is released into cell culture media, where it can be quantified by a coupled enzymatic reaction. LDH catalyzes the conversion of lactate to pyruvate via reduction of NAD+ to NADH. NADH is then used to reduce a tetrazolium salt to a red formazan product, which is then measured spectrophotometrically.

Therefore, this assay was used to evaluate viability of the cells in the epidermal and dermal explants after overnight incubation in serum free media (Fig.4.1). The mean level

of LDH in the media conditioned with SSc epidermis was 0.17±0.05, control epidermis 0.13±0.05, SSc dermis 0.10±0.01, and control dermis 0.18±0.03 while LDH positive control was 1.1±0.02. The values for samples were between 6 and 10 fold lower than the positive control. As expected, there were no significant differences in LDH release between SSc and control samples or between epidermal and dermal explants. This indicates that majority of cells within epidermal and dermal explants were viable, and the concentration of growth factors and cytokines measured in the conditioned media was due to active release process, rather than presence due to release during cell death.

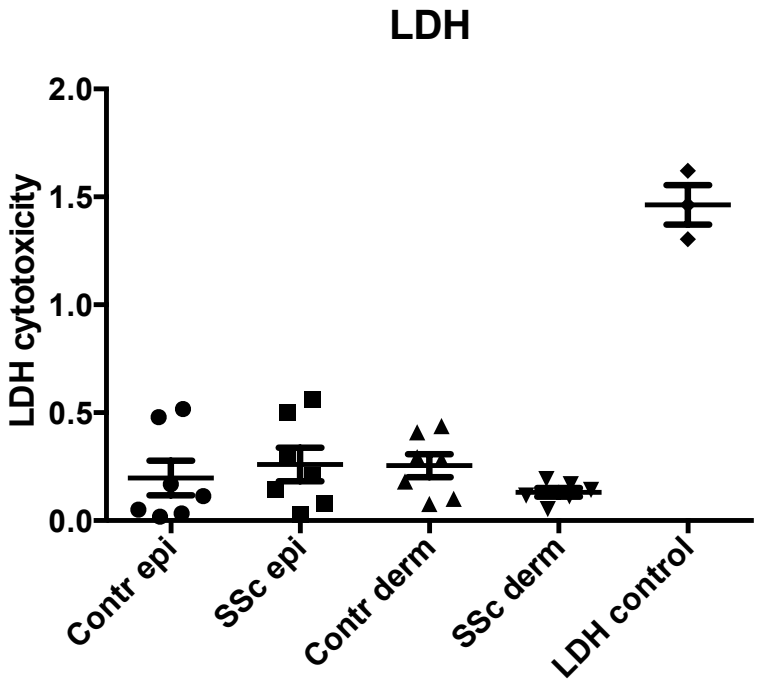


**Figure 4.1. LDH in the conditioned media.**

Skin biopsies were taken from healthy controls (n=17) and patients (n=7). The epidermis was separated from the dermis using trypsin-EDTA and explants were then incubated overnight with serum free media. The graph shows distribution with mean ± SEM concentration of LDH in the conditioned media. Statistical significance was assessed by Mann-Whitney U test. P value was non-significant.

#### 4.3.1.2 Secretion of selected cytokines, chemokines and growth factors into conditioned media by SSc epidermal and dermal explants

After confirming that viability of cells was not significantly afected during overnight incubation, media was subjected to analysis by Luminex or ELISA. In order to establish potential differences in release of inflammatory cytokines and chemokines, angiogenic and profibrotic growth factors, as well as proteins associated with wound healing, conditioned media samples were assayed for classical markers of these processes involved in SSc pathogenesis.

No differences in CCL20 levels in media conditioned were detected in SSc epidermis and dermis samples when compared to control (Fig.4.2a). Similar, MCP-1 epidermal samples were at the same level (Fig.4.2b); however, a 1.4 higher release from control dermis was observed when compared with SSc dermis and showed a trend towards significance ($p=0.2136$). The results of IL-8, another major mediator of inflammatory response, also showed only a trend towards higher release from SSc epidermis than controls with 2.2 fold increase ($p=0.0881$) but, no differences were observed between control and SSc dermal levels (Fig.4.2c).

Unexpectedly, an important cytokine IL-6, previously reported to be overexpressed in SSc skin, revealed only a trend towards significance ($p=0.3889$), although with 4 fold higher release from SSc epidermis than from control; while levels released by control and SSc dermis were similar (Fig.4.2d). However, a few samples classified as outliers (over 200 fold deference from the average) were removed from analysis, reducing statistical power.

Out of all inflammatory mediators tested, the only significant overproduction was observed with S100A9 from SSc epidermis, which showed 2.7 fold increase when compared to control samples ($p<0.05$) (Fig.4.2e). Moreover, this pleiotropic modulator of inflammation in SSc also showed a trend towards enhanced release form SSc dermis compared to controls ($321.5\pm48.79$ and $258.6\pm83.45$, $p=0.1797$).

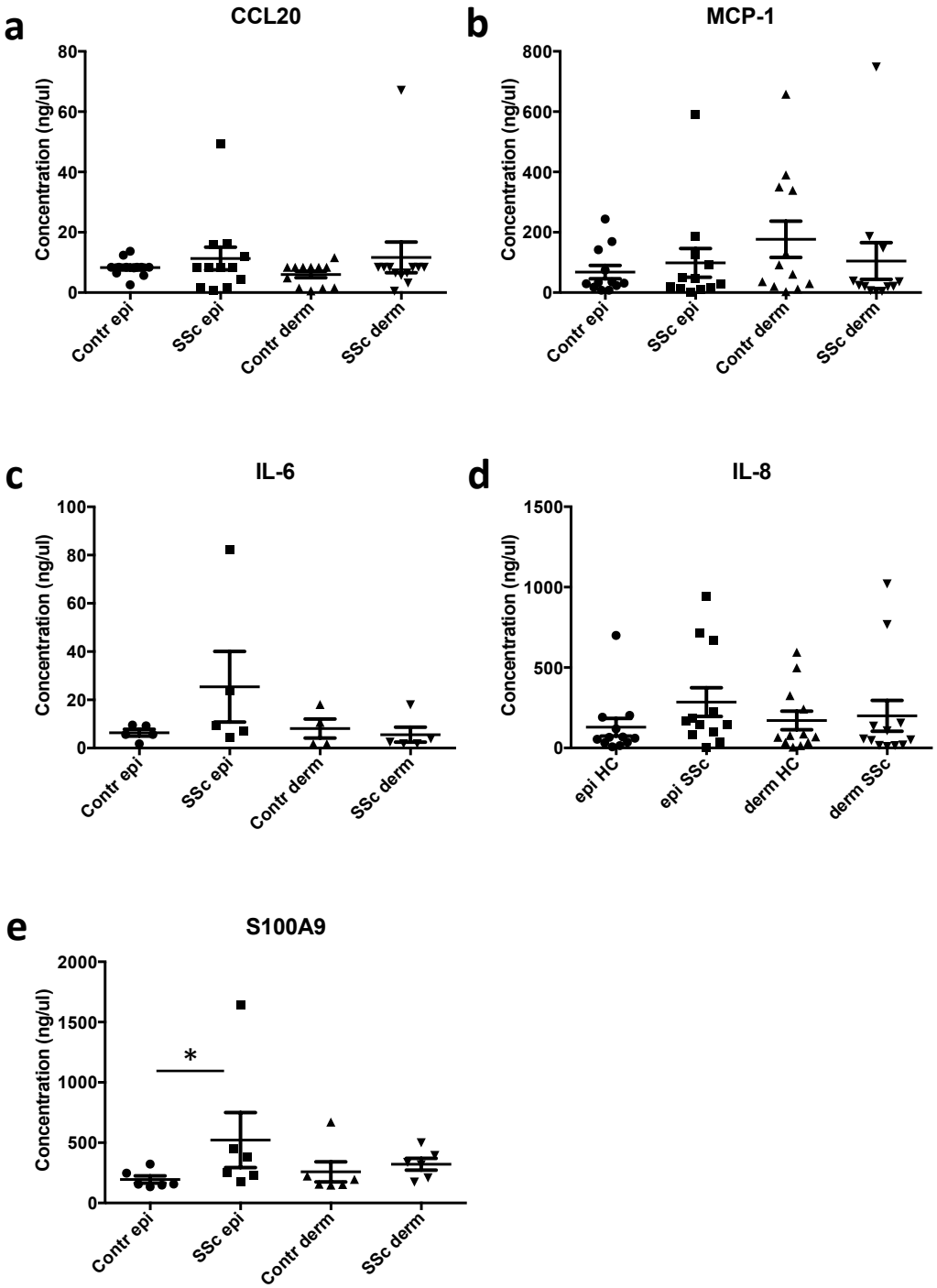


**Figure 4.2. Cytokines and chemokines in the conditioned media.**

Skin biopsies were taken from healthy controls (n=12 for all but IL-6 and S100A9, where n=6) and patients (n=12 for all but IL-6 and S100A9, where n=6). The epidermis was separated from the dermis and explants were incubated overnight with serum free media. The graphs show distribution with mean ± SEM concentration of **(a)** CCL20; **(b)** MCP-1; **(c)** IL-6; **(d)** IL-8; **(e)** S100A9. Statistical significance was assessed by Mann-Whitney test; *$p<0.05$.

Also contradicting previous report (Kawaguchi et al., 2006) showing overexpression of IL-1α by SSc dermal fibroblast, this pro-inflammatory cytokine was undetected in many dermal samples, especially SSc conditioned media. Where the concentration was measured (Fig 4.3a), a trend towards higher release from control dermis with 5 fold increase was observed, ($p<0.05$), while no differences were noted in the levels in the control and SSc epidermal samples.

Levels of another IL-1 family member, IL-1β were in all but 2 SSc epidermal conditioned media below level of detection (Fig.4.3b). More successfully measured was concentration of the IL-1 receptor antagonist, IL-1ra although no significant differences were detected between samples (Fig.4.3c). Interestingly, epidermis seem to be primarily responsible for release of this antagonist with levels in control conditioned epidermis 906.3±431.3 and 1219±537.2 in SSc epidermis compared to much lower in control and SSc dermis (164.2±63.02 and 134.6±70.70).

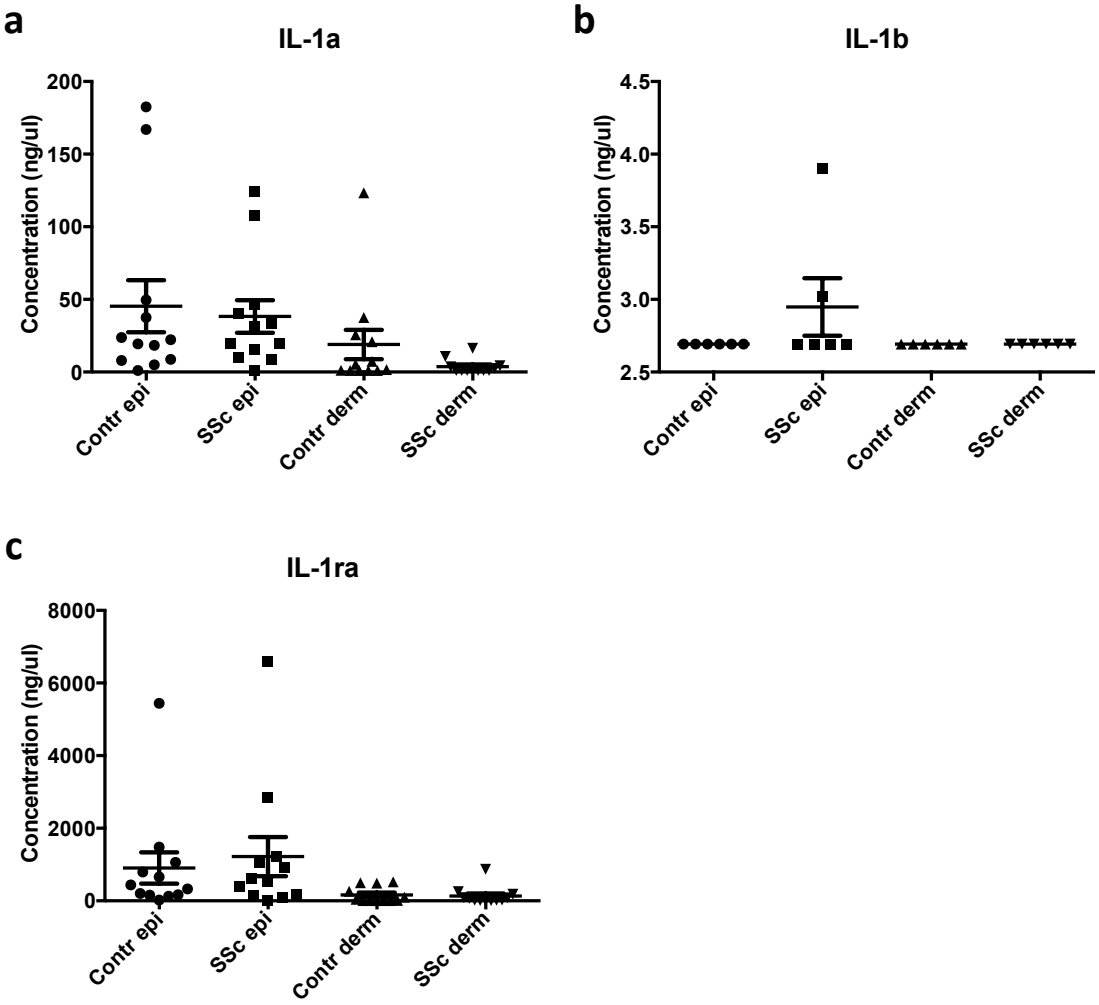


**Figure 4.3. IL-1 family members in the conditioned media.**

Skin biopsies were taken from healthy controls (n=12) and patients (n=12). The epidermis was separated from the dermis using trypsin-EDTA and explants were then incubated overnight with serum free media. The graphs show distribution with mean ± SEM concentration of **(a**) IL-1α; **(b)** IL-1β; **(c)** IL-1ra. Statistical significance was assessed by Mann-Whitney U test. P value was non-significant.

One of the key pro-fibrotic growth factors, CTGF was released in 4.2 higher concentrations from SSc epidermis than from control epidermis ($p<0.05$). Surprisingly, concentrations in samples incubated with SSc dermal explants were below levels of detection, while the mean level in control dermal samples was also low (Fig.4.4a). Whereas, anti-fibrotic HGF release into the media was found to be increased by 4.2 fold in SSc epidermis ($p<0.005$) and 1.6 fold in SSc dermis ($p<0.05$) compared to levels in healthy control subjects (Fig.4.4b). Although, results for the dermal samples were predicted, the significantly increased levels in epidermal samples were unforeseen.

Angiogenic and mitogenic towards fibroblasts, FGF-2 showed a trend towards higher levels in media conditioned by SSc epidermis compared to controls (148.7±26.42 and 117.2±14.76, $p=0.2981$), while no differences were observed between control and SSc dermal samples (Fig.4.4c). No significant differences in levels of secreted VEGF-A, PDGF-AA or G-CSF were observed, when evaluated against levels released by control dermal tissue (Fig.4.4d-f). Though, 1.8 times raised concentrations of VEGF-A ($p=0.14$) and 2.7 times higher PDGF-AA levels ($p=0.2$) found in SSc epidermis conditioned media trended towards significance. GM-CSF in the conditioned media could not be measured as it was below assay detection level.

Since, SSc epidermis demonstrated significantly increased levels of S100A9, CTGF and HGF, those molecules were selected for further investigation.

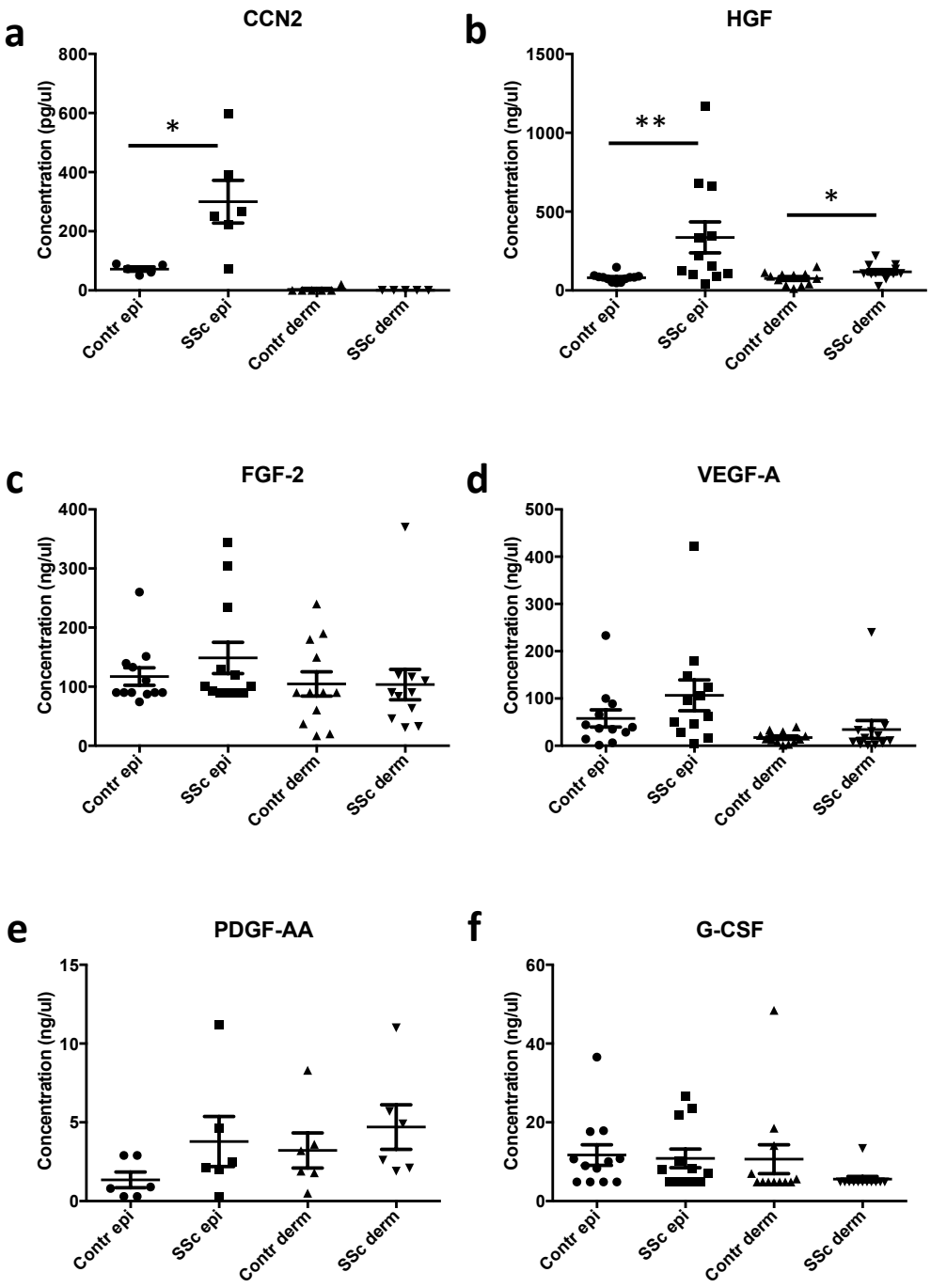


**Figure 4.4. Growth factors in epidermis and dermis conditioned media.**

Skin biopsies were taken from healthy controls (n=12 for all but PDGF-AA where n=6) and patients (n=12 for all but PDGF-AA where n=6). The epidermis was separated from the dermis using trypsin-EDTA and explants were then incubated overnight with serum free media. The graphs show distribution and mean ± SEM for media concentration of **(a)** CTGF; **(b)** HGF; **(c)** FGF-2; **(d)** VEGF; **(e)** PDGF-AA; **(f)** G-CSF. Statistical significance was assessed by Mann-Whitney U test; * $p < 0.05$, ** $p < 0.005$.

### 4.3.2 Detection of CTGF, HGF and S100A9 in the SSc skin using immunohistochemistry

In order to confirm or refute the results of enhanced CTGF, HGF, and S100A9 release into the conditioned media by SSc epidermis, immunohistochemistry was performed on human tissue sections . In healthy controls, positive staining for CTGF (Fig.4.5) was mainly limited to the epidermis, while blood vessels and basal membrane showed only weak staining. In contrast, SSc sections showed pronounced staining in the epidermis, the epithelial/dermal junction (Fig.4.5i) and around blood vessels (Fig.4.5ii). Interestingly, more CTGF was present at epidermal-dermal junction in early stages of disease than established, where it was present in the reticular dermis, especially around vasculature, as well as in the epidermis. This suggests that the epithelial cells express CTGF that accumulates at the epithelial /dermal junction possibly stimulating dermal fibrosis.

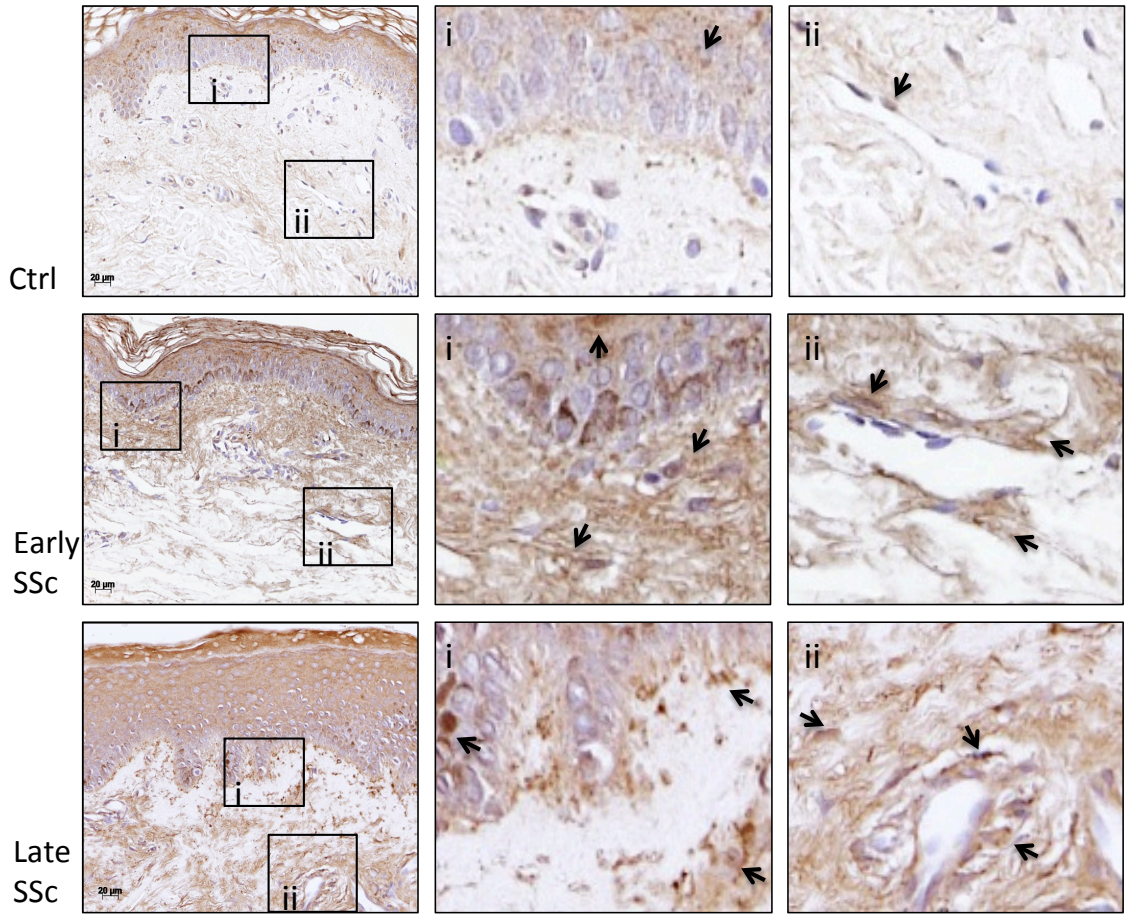


**Figure 4.5. Elevated levels of CTGF in SSc epidermis and at the epidermal-dermal junction.**

Representative images of CTGF staining performed on forearm skin sections taken from early and late SSc patients and controls counterstained with hematoxylin. **(i)** Epidermal-dermal junction, **(ii)** blood vessel. Ctrl-control

The immunohistochemical staining of HGF maintained a very different pattern than CTGF, with stronger staining (Fig.4.6) in healthy epidermis compared to SSc and no changes at the epidermal-dermal junction (Fig.4.6i). However, more HGF positive cells were present in SSc dermis (Fig.4.6ii) than in controls. There was no staining in or around the vasculature. No differences in staining pattern were observed between early and established disease.

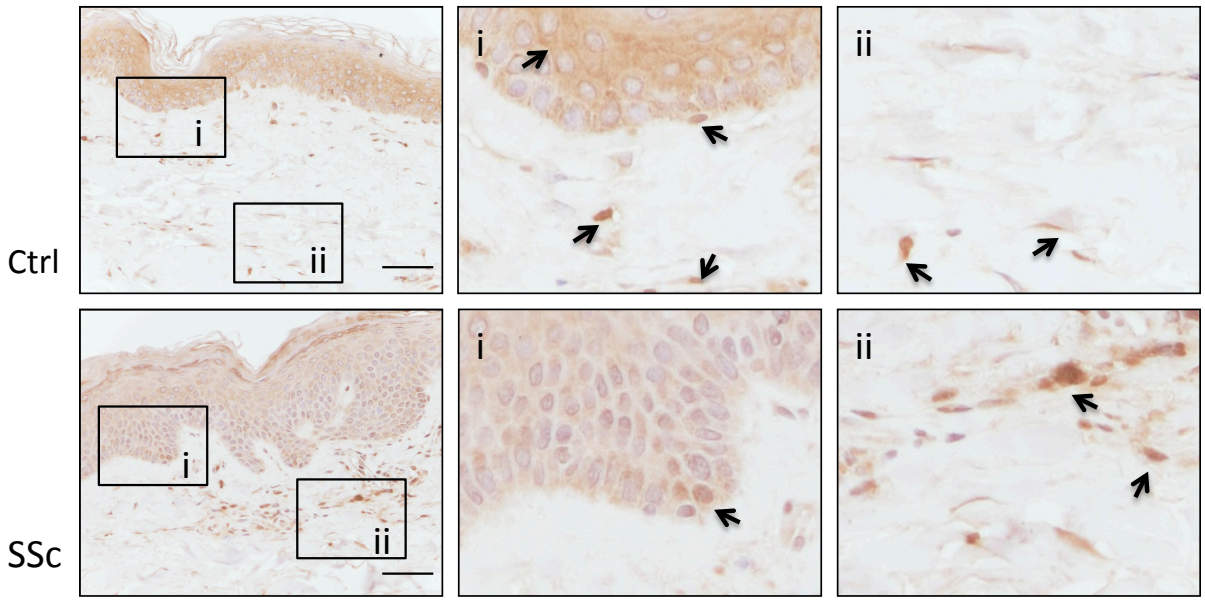


**Figure 4.6. HGF staining in SSc and control skin.**

Representative images of HGF staining performed on forearm skin sections of SSc patients and controls counterstained with hematoxylin. **(i)** Epidermal-dermal junction, **(ii)** dermis. Ctrl-control

The last of the significantly increased molecules in the media conditioned with SSc epidermis was S100A9, a calcium binding protein usually found as a dimer with S100A8. Therefore, immunofluorescent staining was used to determine the location of S100A9 and its heterodimer partner, S100A8 in skin sections. Staining confirmed striking elevated expression of S100A9 in SSc epidermis when compared with control subjects (Fig.4.7). The S100A9 signal was observed throughout SSc epidermis, while in the healthy skin it was present only in some epidermal appendages. S100A8 staining was observed only in the most upper parts of SSc epidermis. Only single cells were double labeled in the dermis in control and disease tissue.

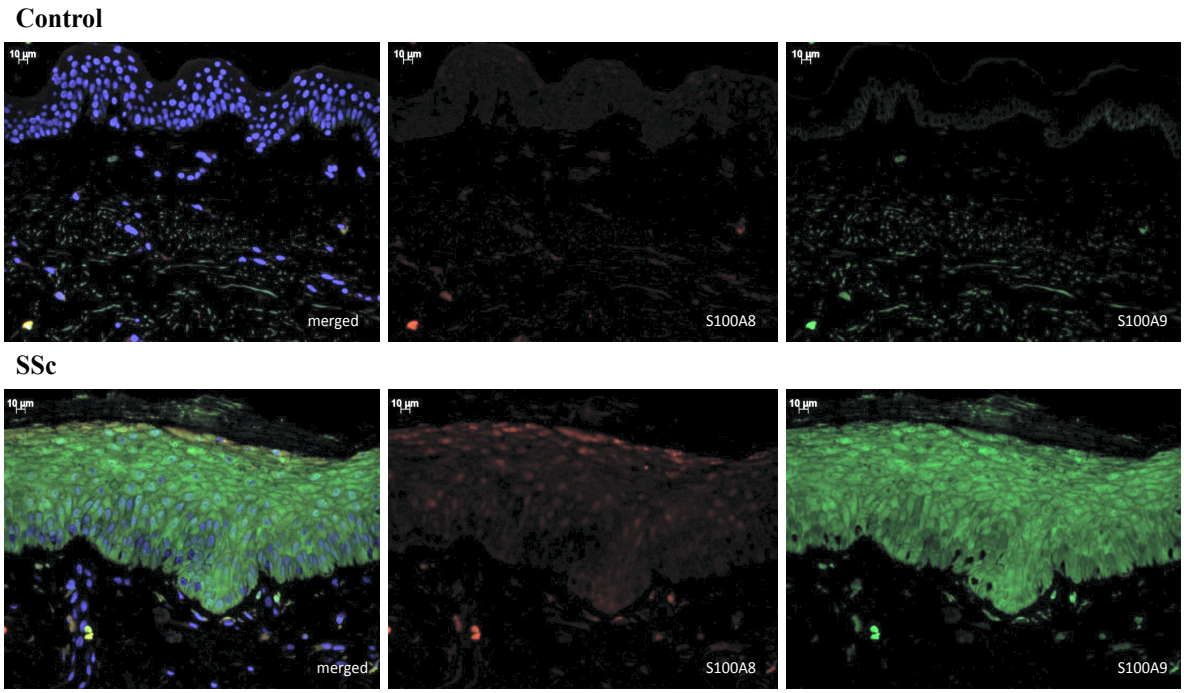


**Figure 4.7. Increased levels of S100A9 staining in the epidermis of SSc patients.**

Representative images of double immunofluorescent staining for S100A8 and S100A9 performed on forearm skin sections of SSc patients and controls.

### 4.3.3 mRNA levels of selected growth factors and cytokines in epidermal blister sheets

Since increase in CTGF and S100A9, but not S100A8, in the SSc epidermis as judged by immunohistochemistry, the levels of the mRNA for these proteins were also investigated. RNA was extracted from epidermal blisters sheets taken from controls (HC n=9) and SSc patients (dcSSc n=24, lcSSc n=8) in order to measure relative expression of CTGF, HGF, S100A9 and proteins closely associated with them mRNA using qPCR. The values were

normalized relative to *TUBB* mRNA expression using the ΔΔCT method and the results presented as mean ± SEM. The one-way Anova test was used to calculate statistical significance, * $p<0.05$.

Results of blister epidermal sheets analysis were consistent with the staining, showing significantly increased in CTGF mRNA levels in dcSSc epidermis compared to controls and lcSSc (both $p<0.05$) (Fig.4.8). The mean levels in control samples (0.2±0.067) and lcSSc (0.17±0.049) were similar, and around 2.5 fold lower than in dcSSc (0.5±0.102).

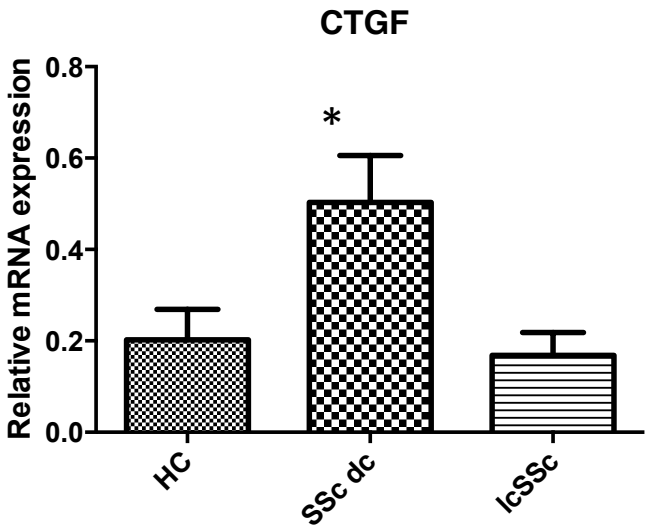


**Figure 4.8. Increased CTGF mRNA in the epidermal blister sheets of dSSc patients.**

Levels of CTGF mRNA were measured in the epidermal blister sheets of SSc patients and controls. The results are presented as mean ± SEM. The one-way ANOVA followed by Tukey's multiple comparisons test was used to calculate statistical significance, * $p<0.05$. HC- control (n=9), dcSSc – diffuse SSc (n=24), and lcSSc- limited SSc (n=8).

To establish if another CCN family member, NOV mRNA is also altered in the SSc epidermis its levels were measured in epidermal blister sheets (Fig.4.9a). Relative mRNA level of control (0.04±0.007) was significantly lower than those from dcSSc patients (0.08±0.009) $p<0.05$). Post hoc analysis did not show any changes between and lcSSc samples (0.05±0.01) and controls or dcSSc samples.

PAI-1 along with CTGF and NOV is often implicated in fibrotic disease, including SSc. However, there are no reports about its levels in SSc epidermis, thus they were measured in the blisters sheets. Results of the qPCR analysis showed that no differences were detected in PAI-1 mRNA level between control, lcSSc and dcSSc samples (Fig.4.9b).

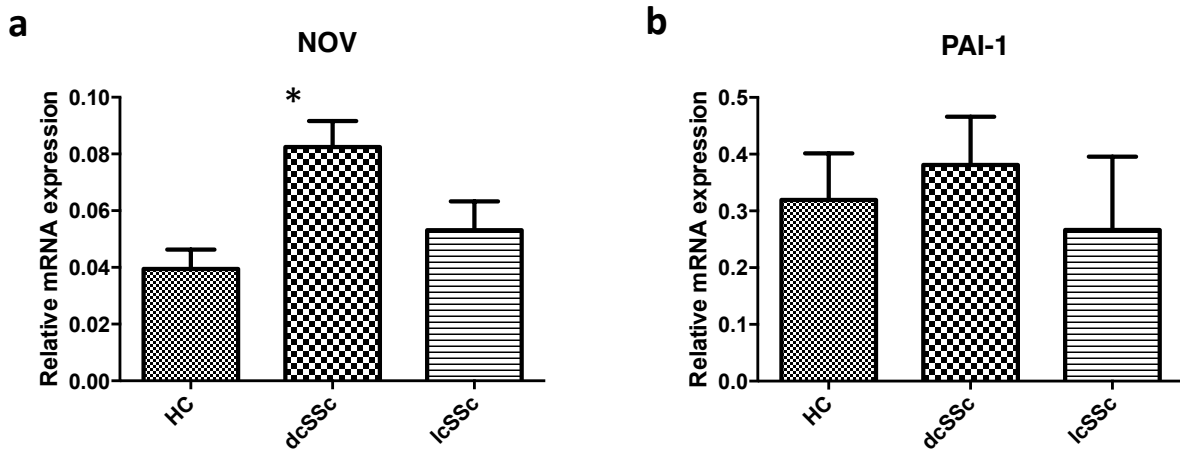


**Figure 4.9. Increased NOV mRNA in the epidermal blister sheets of dSSc patients.**

Levels of **(a)** NOV and **(b)** PAI-1 mRNA were measured in the epidermal blister sheets of SSc patients and controls. The results are presented as mean ± SEM. The one-way ANOVA followed by Tukey's multiple comparisons test was used to calculate statistical significance, * $p<0.05$. HC- control (n=9), dcSSc – diffuse SSc (n=24), and lcSSc- localized SSc (n=8).

In contrast to strong S100A9 staining of SSc epidermis no difference was observed in the levels of S100A9 mRNA in the blister sheets. Equally, no difference was observed in the levels of its heterodimer partner S100A8 mRNA (Fig.4.10). Collectively, these results may indicate that S100A9 protein expression is regulated at post-transcriptional level.

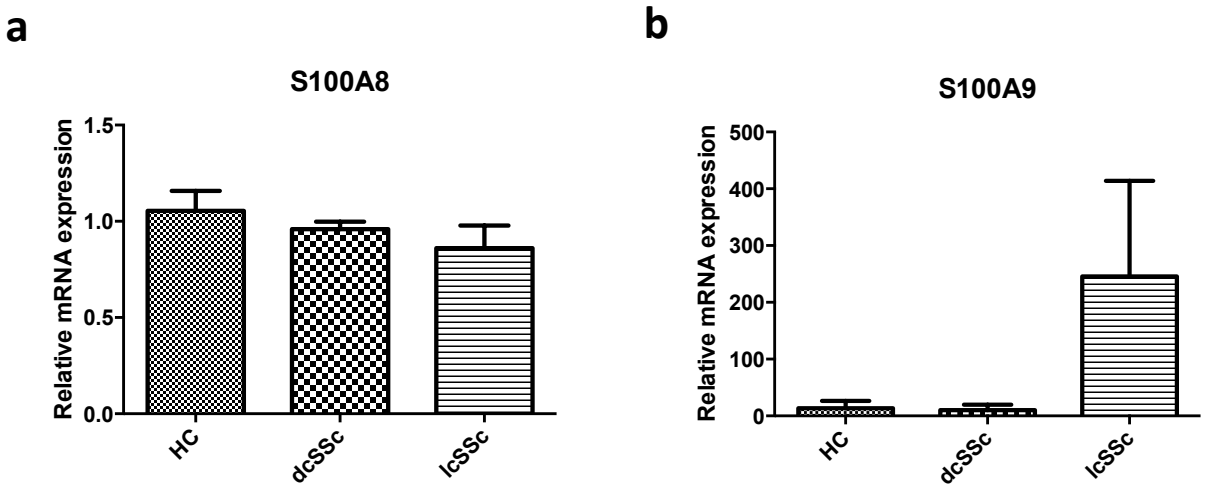


**Figure 4.10. S100A8 and S100A9 mRNA in the epidermal blister sheets of dSSc.**

Levels of **(a)** S100A8 and **(b)** S100A9 mRNA were measured in the epidermal blister sheets of SSc patients and controls. The results are presented as mean ± SEM. The one-way ANOVA test was used to calculate statistical significance. P value was non-significant. HC- control (n=9), dcSSc – diffuse SSc (n=24), and lcSSc- localized SSc (n=8).

TLR4, which transduce signals from bacterial lipopolysaccharide (LPS), as well as endogenous DAMPs including S100A9, was recently shown to be overexpressed in SSc skin and make fibroblasts more responsive to pro-fibrotic stimulation by TGF-β1 (Bhattacharyya et al., 2013). However, analysis of TLR4 mRNA in epithelial blister sheets showed it to be present at similar levels in control and both subsets of SSc (Fig.4.11).

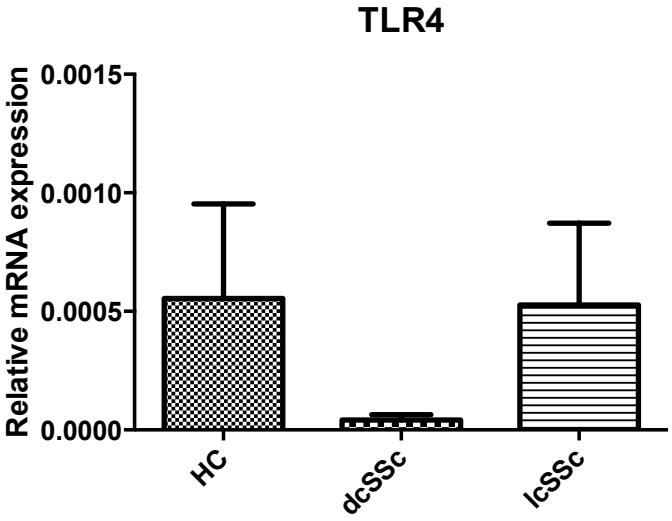


**Figure 4.11 TLR4 mRNA in the epidermal blister sheets of dSSc patients.**

Levels of **(a)** S100A8 and **(b)** S100A9 mRNA were measured in the epidermal blister sheets of SSc patients and controls. The results are presented as mean ± SEM. The one-way ANOVA test was used to calculate statistical significance. P value was non-significant. HC- control (n=9), dcSSc – diffuse SSc (n=24), and lcSSc - localized SSc (n=8).

Although, increase in the IL-6 mRNA levels in SSc dermal fibroblast was reported (Khan 2012), there is no data available on IL-6 mRNA levels in the epidermis or epidermal keratinocytes of SSc patients. Previous report from our group showed extensive IL-6 staining in SSc epidermis and to determine if its production is also increased on the mRNA level its quantities were assessed in the epithelial sheets. The results indicated that IL-6 relative mRNA levels in dcSSc were trending towards lower (p=0.2) when compared with levels in control blister sheets, or even lcSSc (Fig.4.12). However, the quantity of mRNA does not have to be parallel to the protein levels. Due to posttranslational events even despite the normal levels of gene copies the protein may be significantly elevated.

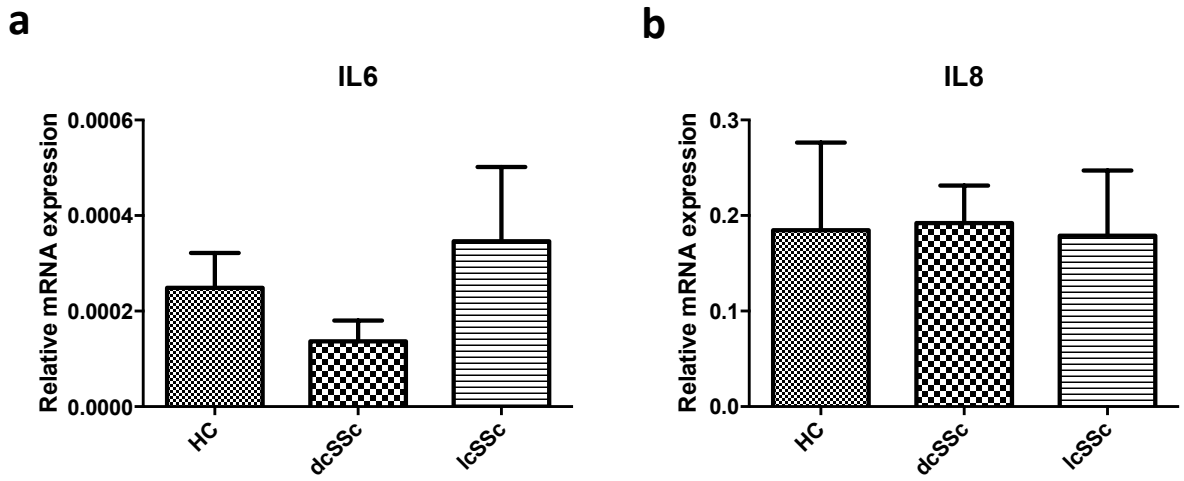


**Figure 4.12. IL-6 and IL-8 mRNA in the epidermal blister sheets of SSc patients.**

Levels of **(a)** IL-6 and **(b)** IL-8 mRNA were measured in the epidermal blister sheets of SSc patients and controls. The results are presented as mean ± SEM. The one-way ANOVA test was used to calculate statistical significance. P value was non-significant. HC- control (n=9), dcSSc – diffuse SSc (n=24), and lcSSc - localized SSc (n=8).

Finally, HGF the last soluble factor identified by conditioned media study to be released at higher concentrations from SSc epidermis. Its mRNA was undetectable in the epidermal sheets of both patients and controls (data not shown), suggesting that the increased HGF protein released from epidermal explants is not being transcribed locally but most likely is produced by dermal fibroblasts.

### 4.3.4 Effect of S100A9 on dermal fibroblasts.

As ELISA on explant conditioned media and histology on skin sections have shown S100A9 to be upregulated in the epidermis of SSc patients, the next step was to establish S100A9 effects on dermal fibroblasts. In particular, I was interested in possible changes to fibroblast migration, proliferation and viability, as well as CTGF expression, as these could impact on pro-fibrotic phenotype of fibroblasts. Therefore, serum starved (DMEM containing 1% FBS) dermal fibroblasts from controls (n=4) and SSc patients (n=4) were incubated with recombinant human S100A9 (rhS100A9) (Sino Biological).

In order to assess impact of S100A9 on viability of fibroblasts, prior to addition of rhS100A9 cells were incubated with mitomycin C (10µg/ml) to stop cell proliferation. After 72hrs the crystal violet method was used to compare differences in cell numbers between control and SSc fibroblasts treated with different concentrations of S100A9. The results revealed that S100A9 did not have any significant effect on control and SSc fibroblast viability when compared with untreated cells (Fig.4.13a).

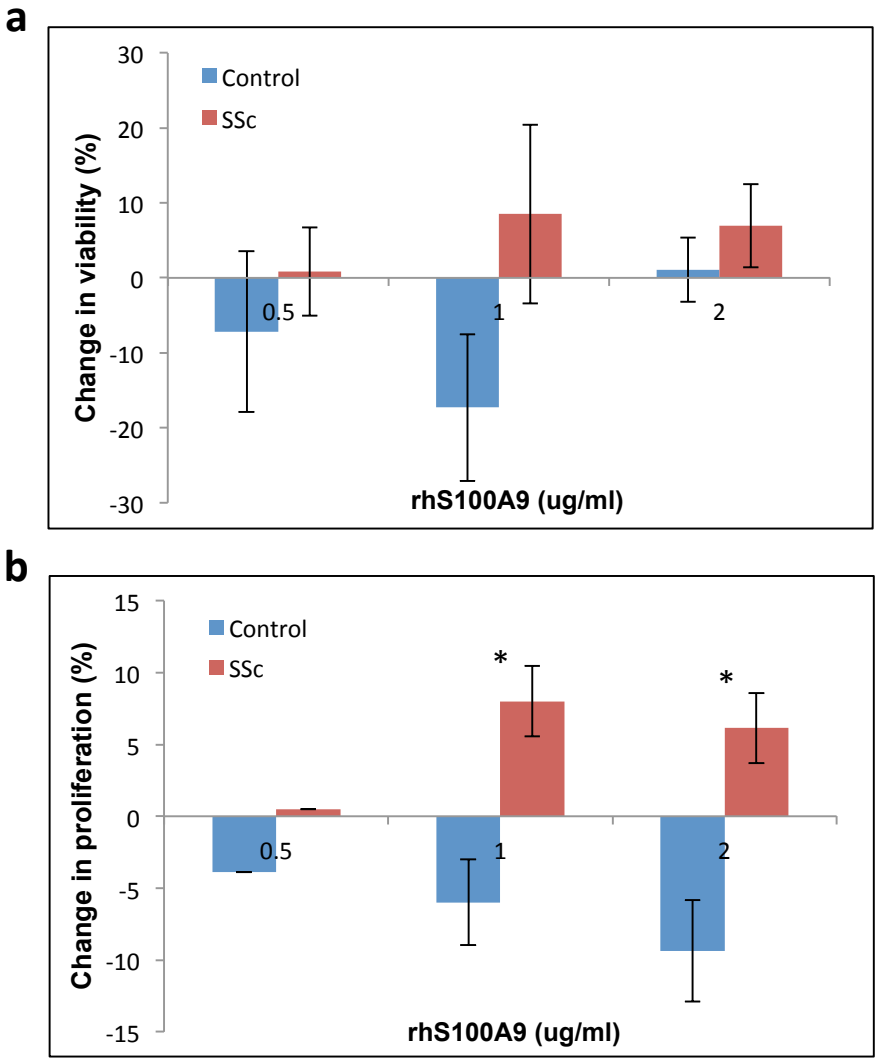


**Figure 4.13. Effect of S100A9 on viability and proliferation of dermal fibroblasts.**

Dermal fibroblasts were incubated with rhS100A9. **(a)** Viability and **(b)** proliferation were measured in dermal fibroblasts of SSc patients and controls, and normalised against untreated samples. The results are presented as mean ± SEM of three independent experiments. The student's t- test was used to calculate statistical significance, * $p<0.05$.

The proliferation rate was assessed 48hrs after the treatment with rhS100A9 using Proliferation Assay (Promega), and revealed differences in response between control and SSc fibroblasts (Fig.4.13b). The percentage change in proliferation as compared to untreated samples showed that, addition of S100A9 at concentrations 1µg/ml and 2µg/ml induced increased proliferation in SSc fibroblast ($p<0.05$), while in control fibroblast it had an opposite effect and decreased proliferation. That difference in response of SSc and control fibroblasts was significant at 1µg/ml and 2µg/ml of S100A9 ($p<0.05$).

For in-vitro scratch assay fibroblasts from healthy controls (n=3) and SSc patients (n=3) were pretreated with mitomycin C (10µg/ml), to assure that the scratch closure is due to migration rather that proliferation, and incubated with rhS100A9. The scratch was assessed after 48 hours and presented as percentage of scratch closure (Fig.4.14). S100A9 at 0.5µg/ml enhanced migration of the fibroblasts from both control and SSc patients ($p<0.05$, $p<0.005$) compared to unstimulated cells. Moreover, this increased migration was also observed in SSc fibroblasts stimulated with 2µg/ml ($p<0.005$), but not the control fibroblasts.

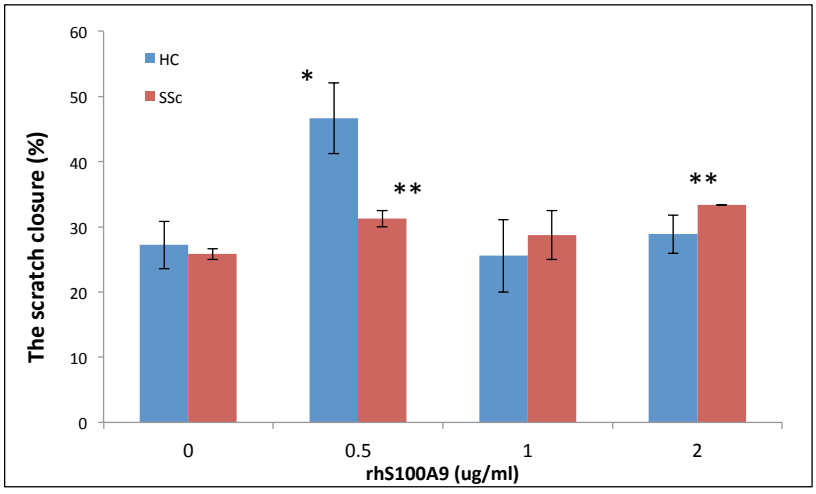


**Figure 4.14. Effect of S100A9 on migration of dermal fibroblasts.**

Dermal fibroblasts were incubated with rhS100A9 migration was measured in dermal fibroblasts of SSc patients and controls. The results are presented as mean ± SEM of three independent experiments. The student's t- test was used to calculate statistical significance, *$p<0.05$, **$p<0.005$.

By stimulation of fibroblast proliferation and migration, S100A9 may take an active role in propagation of fibrotic changes in the SSc skin. To further investigate consequences of increased concentrations of S100A9 in SSc skin on fibroblast, the effect on production of fibrotic markers by dermal fibroblasts was evaluated. Monolayers of dermal fibroblasts from controls (n=3) and SSc patients (n=3) treated with S100A9 for 24hrs were lysed for mRNA, and those treated for 48hrs were lysed for proteins. Western blots revealed that production of CTGF, after incubation with S100A9, was increased in SSc fibroblasts (p=0.05) but not in controls, when normalised against GAPDH (Fig.4.15).

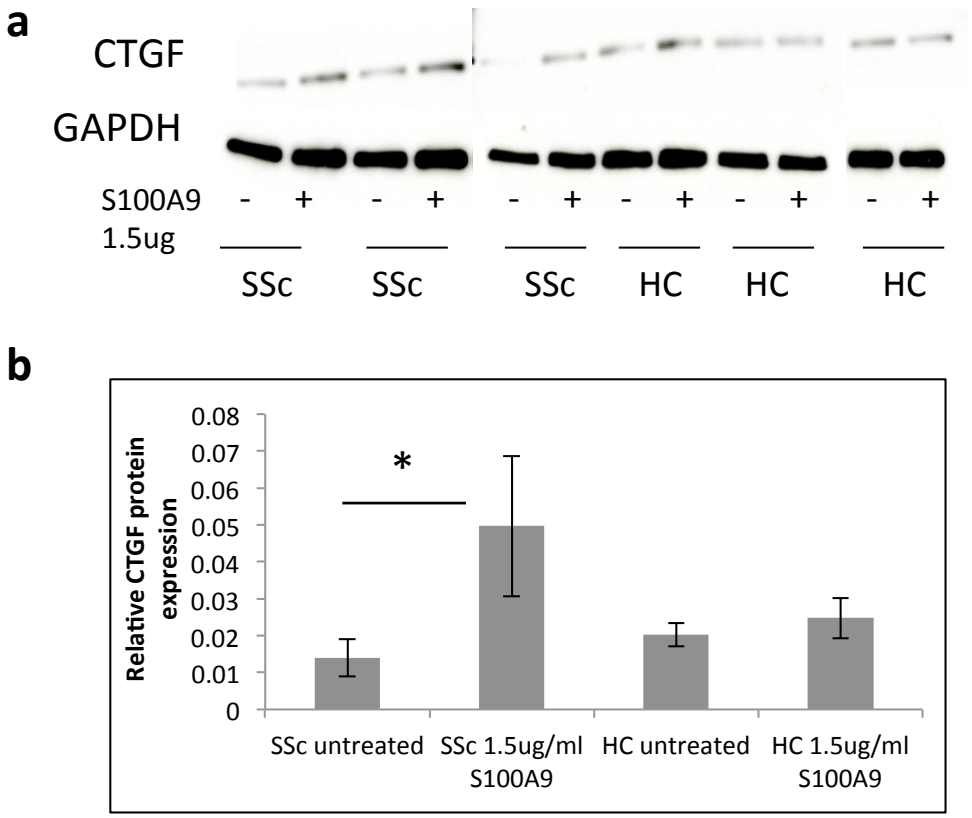


**Figure 4.15. CTGF production by dermal fibroblasts in response to S100A9.**

Dermal fibroblasts were incubated with rhS100A9. **(a)** Membranes were probed with anti- CCN2 antibodies to detect CCN2 levels in cell lysates after 48hour treatment. Anti-GAPDH antibody was used as a loading control. (**b**) Densitometry of the western blot was measured and normalised against GAPDH. Each panel represents the results of two independent experiments. Data was presented as mean ± SD. Statistical significance was assessed by student's t-test; *p≤0.05.

However, the results of type I collagen and α-SMA expression after incubation with S100A9 were not consistent between different primary cell strains (Fig.4.15). Type I collagen was detected only in a few strains, and in those strains it was upregulated by S100A9. α-SMA expression either increased after treatment with S100A9 or did not change. Interestingly, in the group where there were no differences in α-SMA expression after S100A9 treatment, the basal levels of α-SMA were high. This may suggest that those cells might have been already activated despite serum starvation, and could not be further stimulated by addition of S100A9.

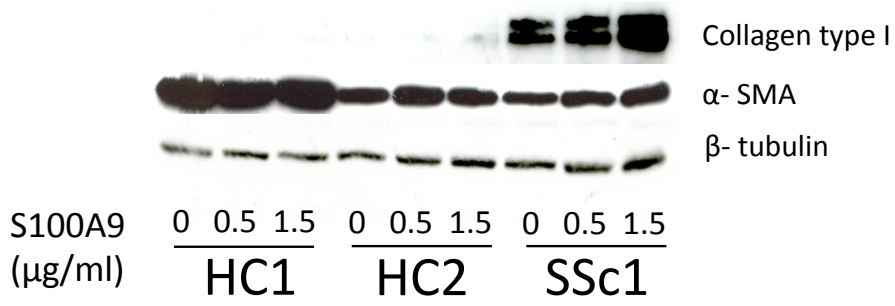


**Figure 4.16. Collagen and α-SMA production in response to S100A9.**

Dermal fibroblasts were incubated with rhS100A9. Membranes were probed with anti- collagen type I and α-SMA antibodies to detect their levels in cell lysates after 48hour treatment. β-tubulin was used as a loading control. Images represent the results of two independent experiments.

As CTGF expression is mainly regulated at the transcription levels, to confirm that S100A9 induces increase in CTGF protein, the mRNA levels were measured after 24hr incubation with S100A9. Due to large inter run variability between experiments the fold change from basal levels was calculated. The results of the analysis confirmed S100A9 enhancing effect on CTGF production (Fig.4.17). A significant increase was observed in response of both control and SSc fibroblasts. The stimulating effect of S100A9 in SSc cells was seen at 0.5µg/ml and 1.5µg/ml ($p=0.01$ and $p= 0.04$ respectively), while control cells required higher concentration ($p<0.05$). This may be due to the higher levels of TGF-β in the SSc skin that could sensitise cells to release CTGF in response to S100A9 so lower levels of S100A9 are required in comparison to control fibroblasts.

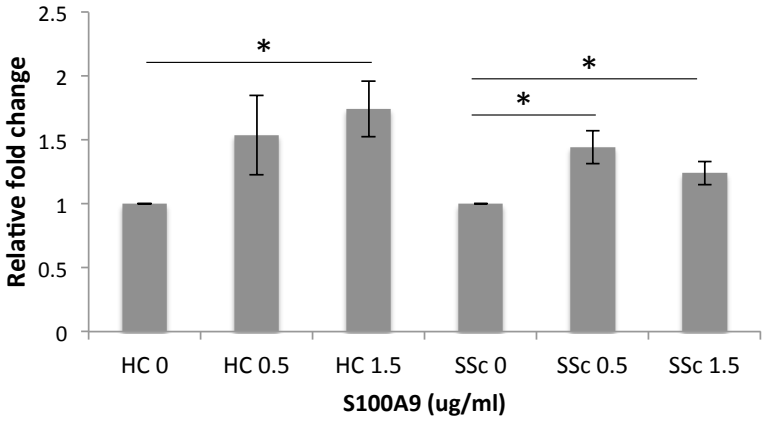


**Figure 4.17. Effect of S100A9 on CTGF mRNA levels in dermal fibroblasts.**

Dermal fibroblasts were incubated with rhS100A9. Relative expression of CTGF mRNA after 24hr treatment was measured with qPCR. The values were normalized relative to *TBP* mRNA expression using the ΔΔCT method. The results are presented as mean ± SEM of fold change form basal expression. The student's t-test was used to calculate statistical significance, * $p \leq 0.05$.

Increased levels of collagen type I are also often seen in fibrosis; therefore its mRNA levels were assessed in dermal fibroblast after stimulation with S100A9. However, in contrast to CTGF, no significant differences were seen between treated and untreated fibroblasts (Fig.4.18).

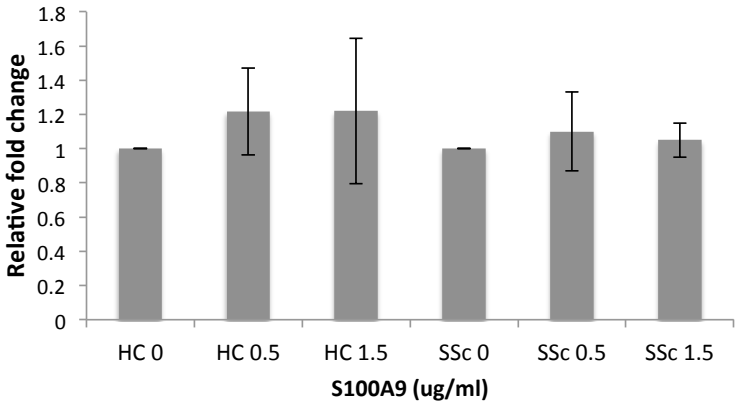


**Figure 4.18. Effect of S100A9 on type I collagen mRNA levels in dermal fibroblasts.**

Dermal fibroblasts were incubated with rhS100A9. Relative expression of collagen type I mRNA after 24hr treatment was measured with qPCR. The values were normalized relative to *TBP* mRNA expression using the ΔΔCT method. The results are presented as mean ± SEM of fold change form basal expression. The student's t- test was used to calculate statistical significance. P value was non-significant.

As described in the introductory chapter of this thesis, IL-6 and IL-1α are key pro-inflammatory cytokines. In addition IL-1α plays critical role in maintaining cross talk between keratinocytes and fibroblasts. Therefore, it was important to establish if S100A9 influences mRNA levels of these cytokines. Considerable differences were observed in the response to the recombinant S100A9 between different strains of primary dermal fibroblasts, especially controls. However, even despite this variability a clear trend towards increased IL-6 was noted in SSc fibroblasts incubated with 0.5μg/ml of S100A9 (p=0.07) (Fig.4.19a). However, no changes were seen in IL-1α mRNA levels (Fig.4.19b).

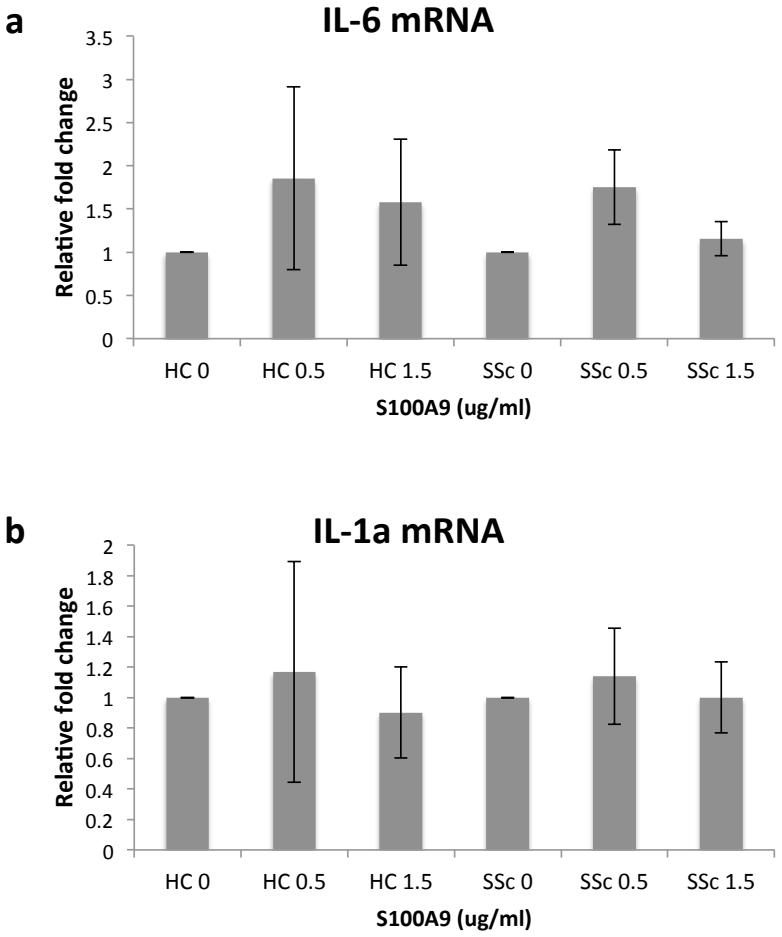


**Figure 4.19. Effect of S100A9 on IL-6 and IL-1α mRNA levels in dermal fibroblasts.**

Dermal fibroblasts were incubated with rhS100A9. Relative expression of IL-6 and IL-1α mRNA after 24hr treatment was measured with qPCR. The values were normalized relative to *TBP* mRNA expression using the ΔΔCT method. The results are presented as mean ± SEM of fold change form basal expression. The student's t- test was used to calculate statistical significance. P value was non-significant.

After studying the response of fibroblasts to stimulation of S100A9 it was important to find out which receptor is responsible for the downstream signaling. I hypothesized that the TLR4 pathway is involved in the process. Therefore an experiment was set up to establish if inhibition of TLR4 would block S100A9 mediated CTGF release. Indeed a selective TLR4 inhibitor TAK-242 TLR4 significantly reduced CTGF mRNA levels in cells stimulated with S100A9 ($p<0.05$) (Fig. 4.20)

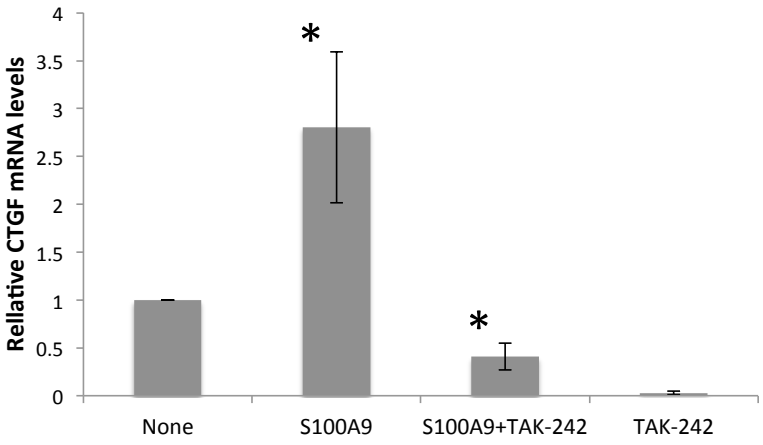


**Figure 4.20. Effect of TLR4 inhibition on S100A9 mediated CTGF mRNA levels in dermal fibroblasts.**

Dermal fibroblasts were incubated with TAK-242 (1μM) prior to stimulation with rhS100A9 (1.5ug/ml). Relative expression of CTGF mRNA after 6hr treatment was measured with qPCR. The values were normalized relative to *TBP* mRNA expression using the ΔΔCT method. The results are presented as mean ± SEM of fold change form basal expression. The student's t- test was used to calculate statistical significance (* $p\leq0.05$).

Similar effect of the TLR4 inhibitor on CTGF secretion upon stimulation with S100A9 was also observed on the protein level (Fig. 4.21).

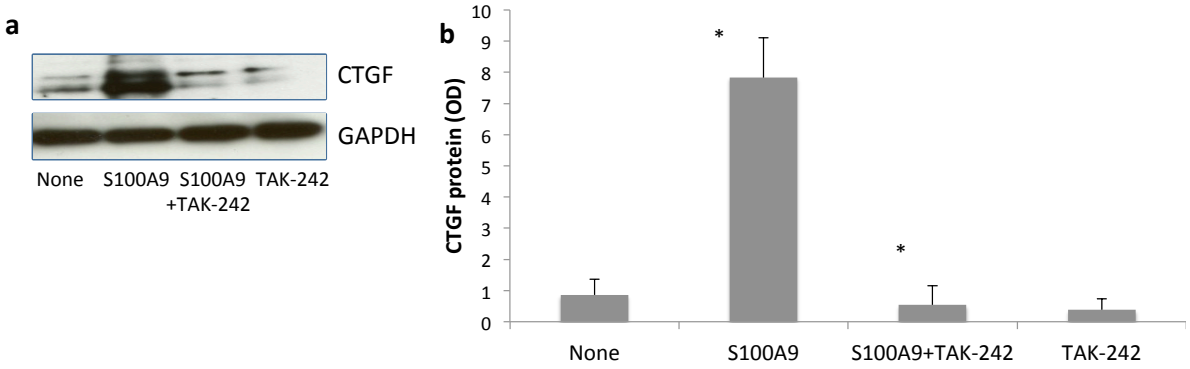


**Figure 4.21. Effect of TLR4 inhibition on S100A9 mediated CTGF levels in dermal fibroblasts.**

Dermal fibroblasts were incubated with TAK-242 (1μM) prior to 48hrs stimulation with rhS100A9 (1.5ug/ml). **(a)** CTGF in cell lysates (HC n=3, SSc n=3). **(b)** Quantitative analysis of the Western blot normalized against GAPDH. The results are presented as mean ± SEM. The student's t- test was used to calculate statistical significance, * $p \leq 0.05$.

### 4.3.5 Effect of S100A9 on keratinocytes.

Although, the hypothesis was that dermal fibroblasts are the main target of S100A9 released by SSc keratinocytes, its autocrine effect is also possible. In order to establish if S100A9 affects proliferation or differentiation of keratinocytes, or their CTGF and IL-6 release, these processes were assessed in HaCaT cells (due to difficulties with access to primary keratinocytes) stimulated with the recombinant protein.

Crystal violet method was used to establish differences in proliferation in response to incubation with S100A9. However, no differences were observed in cell number between treated and untreated cells after 48hrs incubation (Fig.4.22).

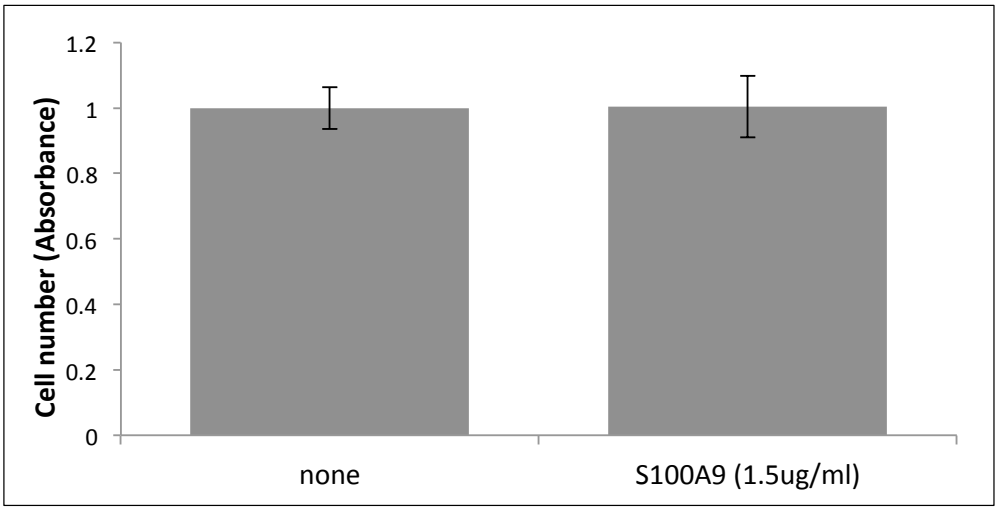


**Figure 4.22. Effect of S100A9 on proliferation of HaCaT cells.**

HaCaT cells incubated with rhS100A9 for 48hr and proliferation was measured using crystal violet method. The results are presented as mean ± SEM of three independent experiments. The t-test was used to calculate statistical significance. P value was non-significant.

The proliferation investigation was followed by an examination of effect S100A9 on keratinocytes differentiation, which could explain changes in the differentiation program of SSc keratinocytes. However, despite previous reports on S100A9 affecting differentiation of keratinocytes (Thorey et al., 2001, Voss et al., 2011), staining of HaCaT cells treated with 1μg/ml of S100A9 for 48hrs, did not reveal any changes in the expression of involucrin and loricrin when compared to untreated cells (data not shown). Though, for that effect a prolonged exposure to S100A9 might be required. Additionally, primary cell strains could behave differently to an immortal HaCaT line, and organotypic 3D cultures of the epidermis might be a better model than monolayer.

Next, I wanted to establish if stimulation of HaCaT cells with S100A9 is able to induce transcription of CTGF gene. Although, TGF-β used as positive control as expected induced increase in CTGF mRNA levels ($p<0.05$), no differences were observed with HaCaT cells stimulated with S100A9, when compared with untreated cells (Fig.4.23).

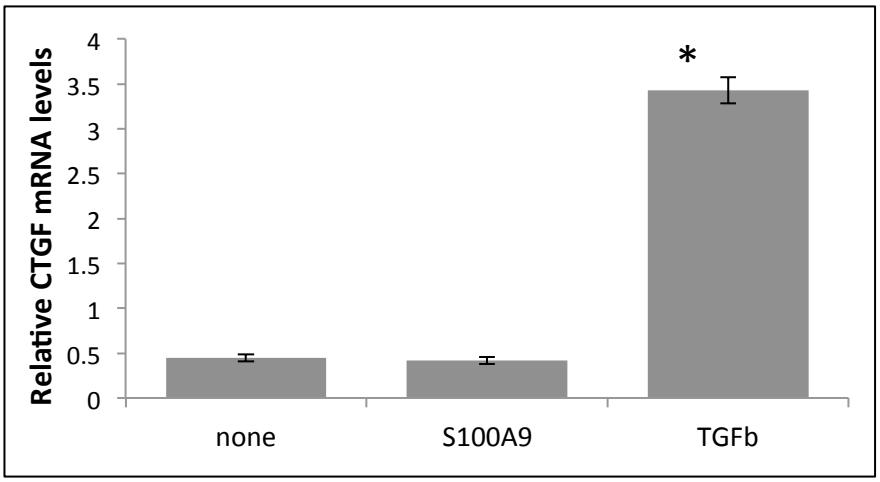


**Figure 4.23. Effect of S100A9 on CTGF mRNA levels in HaCaT cells.**

HaCaT cells were incubated with rhS100A9. Relative expression of CTGF mRNA after 24hr treatment was measured with qPCR. The values were normalized relative to *TBP* mRNA expression using the ΔΔCT method. The results are presented as mean ± SEM of two independent experiments. The student's t- test was used to calculate statistical significance, * $p<0.05$.

Then, I decided to look if S100A9 can induce expression of pro-inflammatory cytokines such as IL-6. The results of ELISA performed on the media taken from HaCaT cells treated with S100A9 showed a trend ($p=0.06$) towards increased release of IL-6 by cells treated with S100A9. As expected incubation of HaCaT cells with LPS, which was used as a positive control, induced over 2 fold increase ($p<0.05$) in released IL-6 compared to both untreated cells and cells treated with S100A9 (Fig. 4.24).

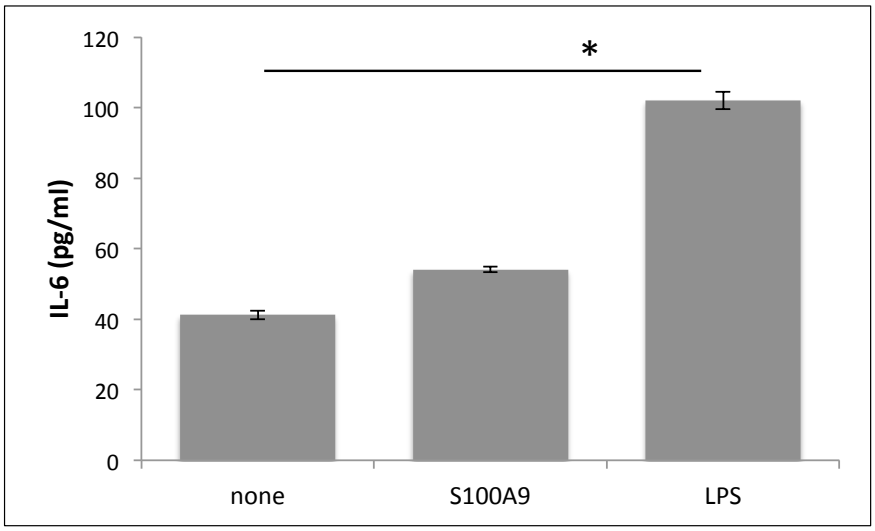


**Figure 4.24. Effect of S100A9 on IL-6 release from HaCaT cells.**

HaCaT cells were incubated with rhS100A9 for 24hr. IL-6 was measured in conditioned media using ELISA. The results of two independent experiments are presented as mean ± SEM. The student's t- test was used to calculate statistical significance, * $p<0.05$.

Therefore, the data presented above supports the initial hypothesis, that in the context of SSc and fibrosis in general, S100A9 has mainly paracrine effect on fibroblast rather than autocrine.

## 4.4 Discussion

One of cardinal features of SSc is skin fibrosis, which is linked to severity and prognosis of the disease. Significant research has focused on the role of the dermis in SSc pathogenesis. Despite the fact that epidermal–dermal interactions are essential for healthy skin, only a limited number of studies have explored the SSc epidermis. Studies from other fibrotic skin diseases have reported the importance of the epidermis in promoting dermal fibrosis (Bellemare et al., 2005, Niessen et al., 2001, Simon et al., 2011), and a report from our group has implicated keratinocytes derived IL-1α as an activator of SSc dermal fibroblasts (Aden et al., 2010). Moreover, data in my previous chapter has demonstrated morphological changes in SSc keratinocytes, which could impact on epidermal interactions in the disease. Therefore, the focus of this chapter was to build upon these observations and explore further the changes in SSc epidermis and identify factors that are most likely to affect the underlying dermal fibroblasts and contribute to disease.

In particular the impact of SSc epidermis on skin inflammation and fibrosis was investigated. Differences in concentrations of secreted molecules, namely FGF-2, CTGF, HGF, G-CSF, VEGF-A, PDGF-AA, IL-1α, IL-1β, IL-1ra, IL6, IL8, CCL20, MCP-1, and S100A9, between normal and SSc epidermis were examined; histological studies with a selected panel of proteins were performed, which were then followed by analysis of the expression of genes encoding these proteins.

One of the explanations for the abnormal differentiation in SSc epidermis, demonstrated in the previous chapter, could be a change in the epidermal-dermal interactions caused by differential expression of growth factors and cytokines. The secretome analysis results revealed that SSc epidermis released significantly higher amounts of CTGF, HGF and S100A9 compared to healthy epidermis, while SSc dermis released significantly higher levels of HGF than healthy dermis (Nikitorowicz-Buniak et al., 2014). The data presented above illustrates the ability of SSc epidermis to produce soluble factors relevant to SSc

pathogenesis and thus the possible importance of SSc epidermis in generation and maintenance of the disease.

CTGF, responsible for stimulation of fibroblasts growth, myofibroblast differentiation (Grotendorst et al., 2004), matrix production and granulation tissue formation, is considered a key factor driving fibrosis in SSc (Frazier et al., 1996). CTGF is overexpressed in the majority of fibrotic diseases including SSc, where its increased serum, plasma and dermal interstitial fluid levels are associated with severity of skin disease and disease duration (Sato et al., 2000). Furthermore, the CTGF gene, primarily regulated by TGFβ at transcriptional level, is overexpressed in SSc skin and lung fibroblasts (de Winter et al., 2008, Shi-Wen et al., 2008). Here I demonstrate that this critical factor for maintenance of fibrosis is also upregulated in SSc epidermis and bound to the ECM at the dermal epidermal junction in early disease.

CTGF levels in SSc epidermal explants conditioned media were significantly higher than healthy controls. Enhanced staining in SSc epidermis, at epidermal-dermal junction and around blood vessels, further strengthened the evidence of increased CTGF production by SSc epidermis and epidermal contribution to the disease. The findings were also confirmed by epidermal blister sheets analysis, which showed a significant increase in CTGF mRNA in SSc. Interestingly, mRNA of another member of CCN family often linked to fibrosis, NOV (CCN3) was also found to be increased in the epidermal blister sheets, suggesting important role of CCN family members in the diseased epidermis.

HGF, produced by mesenchymal cells upon injury becomes cleaved to a mature biologically active form, which binds to the c-Met receptor on epithelial cells and enhances their proliferation and migration to enable wound closure. However, HGF can also act in an autocrine manner, when released by epithelial cells themselves, as in certain types of cancer and extensive skin injury (Nayeri et al., 2006). Keratinocytes overexpressing HGF are hyperproliferative and form thickened epidermis (Hamoen and Morgan, 2002). HGF can be regarded as anti-fibrotic agent due to its ability to suppress collagen synthesis and CTGF in SSc fibroblasts (Bogatkevich et al., 2007, Jinnin et al., 2005, Sherriff-Tadano et al., 2006). Previous works showed enhanced HGF levels in SSc

patients serum (Beirne et al., 2009), increased mRNA levels in SSc skin (Frost et al., 2012), and amplified expression in SSc fibroblasts along with its receptor c-Met (Kawaguchi et al., 2002). Results from our group have demonstrated increased phosphorylation of c-Met in SSc epidermis (Aden et al., 2010).

Data in this report show significantly raised HGF in the conditioned media from SSc epidermis when compared with healthy controls. However, immunostaining and mRNA assay results suggests that keratinocytes might not be an important source of HGF in SSc skin. They indicate that the local fibroblasts might be responsible for the overproduction and keratinocytes are a target. Therefore, it would be useful to compare levels in the media with the levels bound to c-met receptor. Increased presence of this anti-differentiation factor could explain delayed differentiation of SSc keratinocytes.

The results of ELISA performed on conditioned media by epidermal cells also showed that S100A9 is released in higher concentrations by SSc epidermis and suggested keratinocytes as the main sources of the protein in the SSc skin. This was further confirmed by immunofluorescent staining, which revealed S100A9 expression in basal and SSc epidermis, in contrast to just weak basal expression in control epidermis. Only weak staining and no changes in S100A8 expression pattern were found between SSc and control sections. Results of the epidermal blisters sheets analysis further confirmed increase S100A9 mRNA in SSc and no differences in S100A8 mRNA levels, suggesting that in SSc skin S100A9 does not heterodimerise with S100A8.

S100A9 belongs to a family of calcium-binding S100 proteins and was reported to reduce keratinocyte proliferation and increase differentiation (Voss et al., 2011). Its overexpression is seen in suprabasal keratinocytes of inflamed hyperproliferative, abnormally differentiated epidermis like in psoriasis (Benoit et al., 2006); certain types of cancers and inflammatory skin diseases such as lupus erythematosus. Therefore, its high levels might be also responsible for the abnormal differentiation of SSc keratinocytes. Moreover, S100A9 is one of the damage associated molecular pattern (DAMPs) molecules, a danger signal recognised by immune cells and known to be induced in

keratinocytes during epidermal stress (Thorey et al., 2001, Marionnet et al., 2003, Dazard et al., 2003). S100A9 secretion stimulates expression of IL-8, IL-6 and other pro-inflammatory cytokines which then stimulate release of S100A9 (Nukui et al., 2008). Such positive feedback loop further perpetuates pathological changes. These pro-inflammatory properties of S100A9 were investigated in this thesis using the HaCaT cell line, and dermal fibroblasts as targets.

Although, the role of S100A9 in SSc is unknown, recent publications suggest its importance in the development of the disease, and show increased levels of S100A9 in SSc skin and plasma (Xu et al., 2013b), serum, feces and saliva of SSc patients (Andreasson et al., 2011, Giusti et al., 2007). S100A9 has been shown to signal via TLR4 and RAGE, and both pathways contribute to CTGF production. Moreover, overexpression of both RAGE (Davies et al., 2009) and TLR4 (Bhattacharyya et al., 2013) was previously reported in SSc skin. Although, the results presented in this chapter did not show differences in TLR4 mRNA between control and SSc sections, this does not rule out active TLR4 signalling and might be caused by anti-inflammatory therapy, that patients undergo in order to control symptoms of SSc, or the effects of statins, which are widely prescribed to chronic disease patients. Recently, S100A9 was reported to promote lung fibroblast activation via RAGE pathway (Xu et al., 2013a) while enhanced TLR4 signalling seem to sensitize fibroblasts to pro-fibrotic stimulation by TGF-β1, a widely accepted factor in SSc pathogenesis (Bhattacharyya et al., 2013).

Recently S100A8/S100A9 was also reported to be produced by activated keratinocytes of FGF receptor knockout mouse and identified as a key player in development of dermal fibrosis in these mice (Meyer et al., 2011). Moreover, increased S100A8/S100A9 circulatory levels were also shown to associate with SSc lung fibrosis (van Bon et al., 2014). However the exact form of the S100A8 protein was not investigated, thus it is difficult to comment if the SSc pulmonary disease link is related to homo- or heterodimer. Therefore, the direct effect of S100A9 on fibroblasts and keratinocytes in a context relevant to fibrosis was established. The results have shown that HaCaT cells stimulated with S100A9 have increased IL-6 levels. Moreover, S100A9 stimulates

proliferation and migration of SSc dermal fibroblasts, and induces CTGF protein and mRNA in cultured dermal fibroblasts from both SSc and control. This is an important finding suggesting a link between pro-inflammatory and pro-fibrotic pathways that may play role in SSc pathogenesis. It emphasizes the potential for epithelial damage or activation to modulate dermal fibroblast properties by induction of key growth factors, and demonstrates the importance of interactions of epidermal keratinocytes with dermal fibroblasts. However, this mechanism might be responsible for changes in other organs too. Implication of TLR4 depended pathway provides a link between damage signal and involvement of innate immune system profoundly linked to fibrosis.

# Chapter Five

# Results: TGFβ signalling – EMT in SSc skin

## 5.1 Introduction

Presence of active TGFβ signalling in SSc skin and its crucial role in SSc pathogenesis are well documented (Xu et al., 1991, Leask et al., 2002, Rudnicka et al., 1994, Holmes et al., 2001). Enhanced TGFβ1 and TGFβ receptors expression was observed in the skin, including the epidermis of patients with active SSc disease (Higley et al., 1994). TGFβ not only promotes fibrosis on its own, but also induces expression of other pro-fibrotic factors such as ET-1 (Shi-wen et al., 2007), PDGF (Bonner, 2004) and CTGF, all of which are also implicated in SSc disease. CTGF, which is often used as a downstream marker of TGF-β signalling, was shown in previous chapter of this thesis to be overexpressed in SSc epidermis. Its induction is known to be mediated by the TGF-β/Smad pathway (de Winter et al., 2008), which can also stimulate epithelial cells to undergo EMT and acquire the properties of mesenchymal cells (Hoot et al., 2008).

Another process associated with EMT is persistent inflammation, which leads to the release of cytokines into the microenvironment of epithelial cells, triggering their activation, which can then result in their transition into fibroblasts. Originated this way fibroblasts become activated by inflammatory signals and start to produce ECM, which accumulates leading to tissue fibrosis. A link between EMT and lung fibrosis is well established (Kim et al., 2006, Yasukawa et al., 2013), but in case of fibrosis of other organs, such as kidney it is disputable (Iwano et al., 2002). In a previous chapter of this thesis I have demonstrated increased levels of pro-inflammatory S100A9 and IL-6 in the SSc epidermis. Reports of enhanced expression of other inflammatory markers in SSc skin were also published (Koch et al., 1993, Tao et al., 2011).

Therefore, such a pro-inflammatory environment combined with active TGFβ signalling in the SSc epidermis could potentially trigger the EMT process in SSc keratinocytes. Recently, two groups reported evidence for EMT associated changes in SSc skin (Nakamura and Tokura, 2011, Gillespie et al., 2011); however, these reports were not followed by more extensive studies. EMT could link the changes found in the SSc epidermis, with the persistent myofibroblast phenotype seen in the underlying dermis.

Thus in this chapter I have explored whether there is any evidence of SSc epidermal keratinocytes undergoing an EMT process using immunohistochemistry and RT-qPCR techniques.

## 5.2 Results

Studies were performed to determine whether complete or partial EMT might be a feature of abnormal epidermis of SSc patients and contribute to the increased population of myofibroblasts seen in the SSc skin.

### 5.2.1 Expression of phosphorylated Smad2/3 and K14 in SSc skin

Smad dependent TGFβ1 signalling has been implicated in wound healing and fibrosis. Its ability to stimulate production of pro-inflammatory cytokines, ECM synthesis, but also stimulate proliferation and differentiation of fibroblasts, as well as regulation of keratinocytes makes Smad/TGFβ1 an important player in SSc pathogenesis. Previous studies have demonstrated increased TGFβ1 in the skin, but they were mainly focused on the dermis. In order to examine for the presence of Smad signalling in the SSc epidermis, immunohistology was performed on skin sections.

Analysis of sections stained with anti-phospho-Smad2/3 antibodies (red) revealed its co-localisation with nuclear stain DAPI (blue) in SSc skin (Fig.5.1). This nuclear expression was observed throughout the SSc epidermis, apart from the last granular layer, where the protein was stained in the cytoplasm. In contrast, in healthy epidermis no staining was observed in the basal cells, while the supra-basal cells showed cytoplasmic localisation.

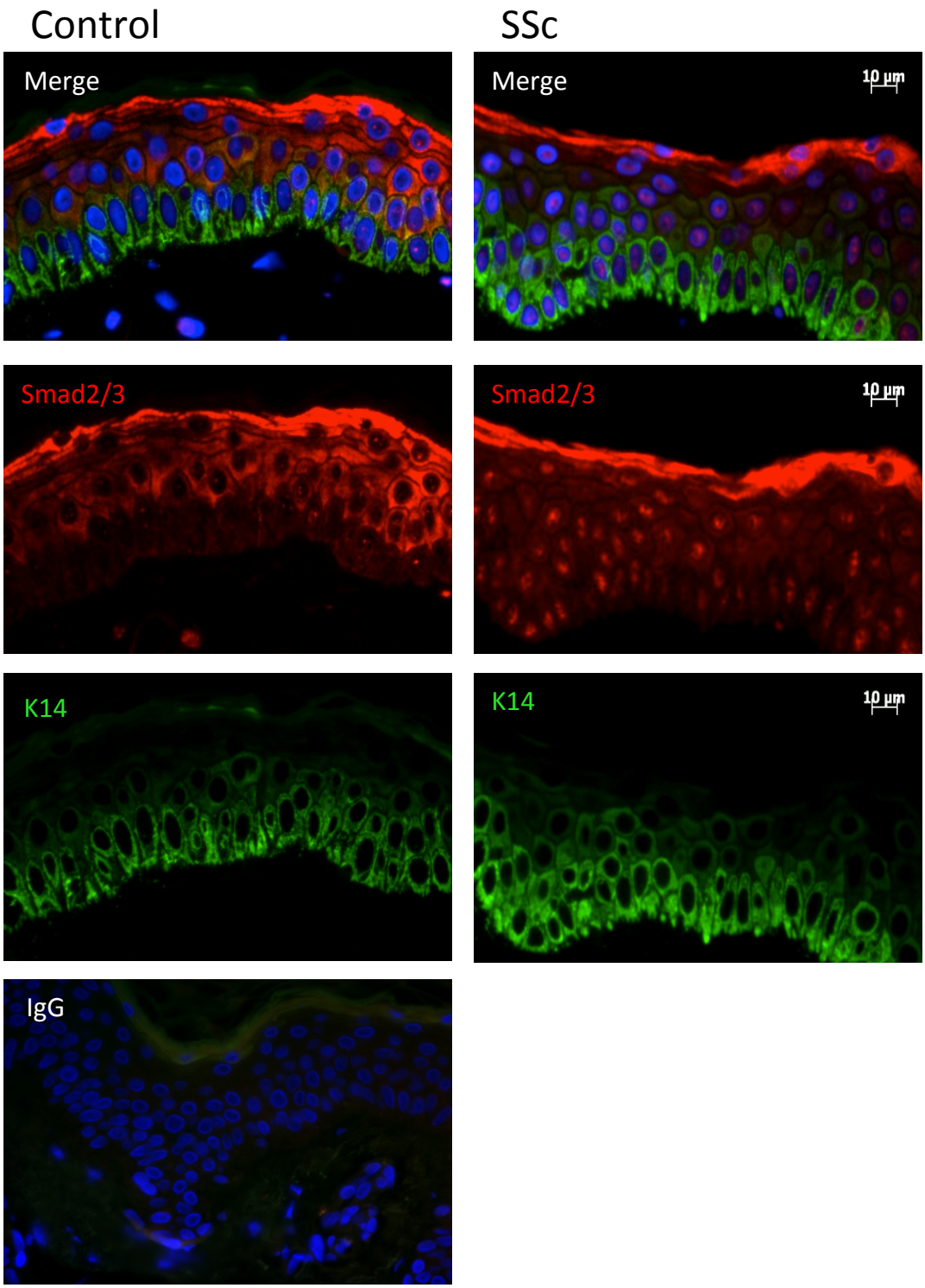


**Figure 5.1. Phosphorylation of Smad2/3 in SSc epidermis.**

Representative images of double immunofluorescent staining performed to detect phosphorylated Smad2/3 (red) and K14 (green) in the epidermis of SSc patients and controls forearm skin sections. DAPI (blue) was used to stain nuclei.

In order to investigate possible cross talk between the SSc epidermis and local dermal fibroblasts, p-Smad2/3 positive cells within the immediately adjacent dermis (Fig.5.2a) and in the epidermis (Fig.5.2b) were examined. A significant increase was noted in the number of p-Smad2/3 positive cells in the SSc papillary dermis from 41.6±7.55 in controls to 69.2±10.94 in SSc ($p<0.05$). However, even more pronounced difference was observed in the number of positive keratinocytes from 31.4±5.12 in controls to 123.6±16.58 in SSc ($p<0.005$).

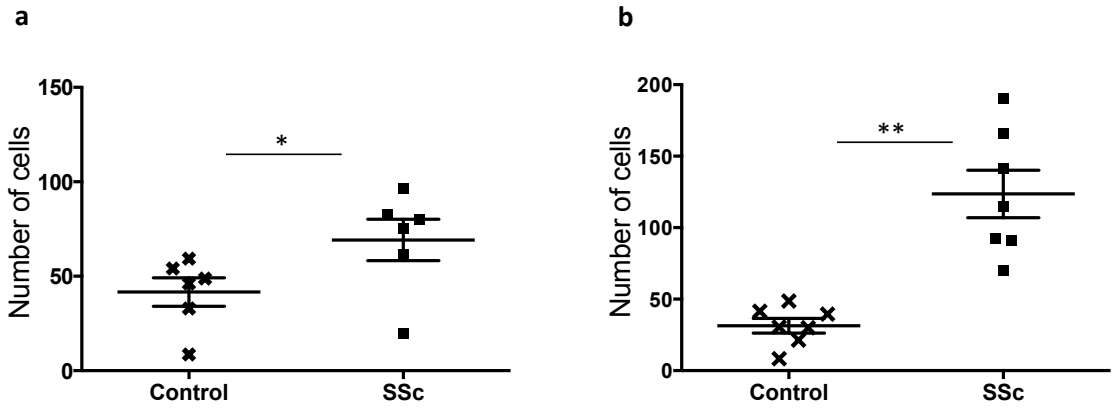


**Figure 5.2. Quantitative analysis of phospho-Smad2/3 staining in SSc skin.**

Cells showing nuclear phospho-Smad2/3 staining were counted and mean was calculated for each section based on average of 5 high magnification fields of view. The graphs show mean± SEM of phospho-Smad2/3 positive cells in **(a)** papillary dermis and **(b)** epidermis of controls (n=6 and 7 respectively) and SSc patients (n=6 and 7 respectively). The Mann-Whitney test was used to calculate statistical significance, * $p<0.05$, ** $p=0.005$.

Such augmented activation of Smad2/3 in the SSc keratinocytes and fibroblasts at the epidermal-dermal junction, suggests that in this area cross- talk between these 2 cell types takes place, but also confirms previous findings of active Smad dependent TGFβ signalling in the SSc skin, including epidermis. Moreover, increased TGFβ signalling in the papillary dermis is consistent with the increased CTGF staining in this area of the early SSc samples, showed in the previous chapter.

### 5.2.2 Modeling EMT in HaCaT cells stimulated with TGFβ

As already mentioned, Smad dependent TGFβ signalling described in the SSc skin (Honda et al., 2012, Gruschwitz et al., 1990, Falanga et al., 1992) can also stimulate keratinocytes to undergo EMT. The origin of myofibroblasts present in the fibrotic tissue seen in SSc is unclear, and the EMT process is given as one of the possibilities of generation of these cells. Therefore, it had to be determined, whether SSc epidermal keratinocytes loose their epithelial phenotype and gain characteristics of mesenchymal cells, thus contributing to increased population of local fibroblasts. In order to validate qPCR assays and protein markers relevant to the process, before moving into experiments with patients' samples, an EMT model was established. Cells of the immortal keratinocyte line, HaCaT were incubated with TGFβ1, a known EMT inducer. The cells were then either used for immunofluorescent staining, or lysed for protein/ mRNA assays. The sections below present the results of these assays.

In order to look at the ability of detection of EMT changes by immunofluorescent staining with in house antibodies, HaCaT cells were incubated with TGFβ1 for 72hr (Rasanen and Vaheri, 2010). As anticipated, morphology assessment by bright field microscopy, revealed altered cell morphology from the typical for keratinocytes cobble shape, into more elongated fibroblast-like phenotype in treated cells (Fig.5.3 BF). Furthermore, cells incubated with TGFβ1 had lower density than the controls, suggesting inhibition of proliferation, as previously described (Munger et al., 1992, Kretschmer et al., 2003).

In addition, immunofluorescent staining with epithelial specific marker Pan-cytokeratin and the mesenchymal cell marker FSP-1 was performed to assess the effect of the treatment on the expression of epithelial and mesenchymal markers. Consistent with changes in shape, TGFβ1 treatment caused decreased expression of Pan-cytokeratin with simultaneous increase in FSP-1 (Fig.5.3). Those results indicate, that as expected, HaCaT cells treated with TGFβ1 started to acquire a mesenchymal phenotype, which is consistent with EMT changes.

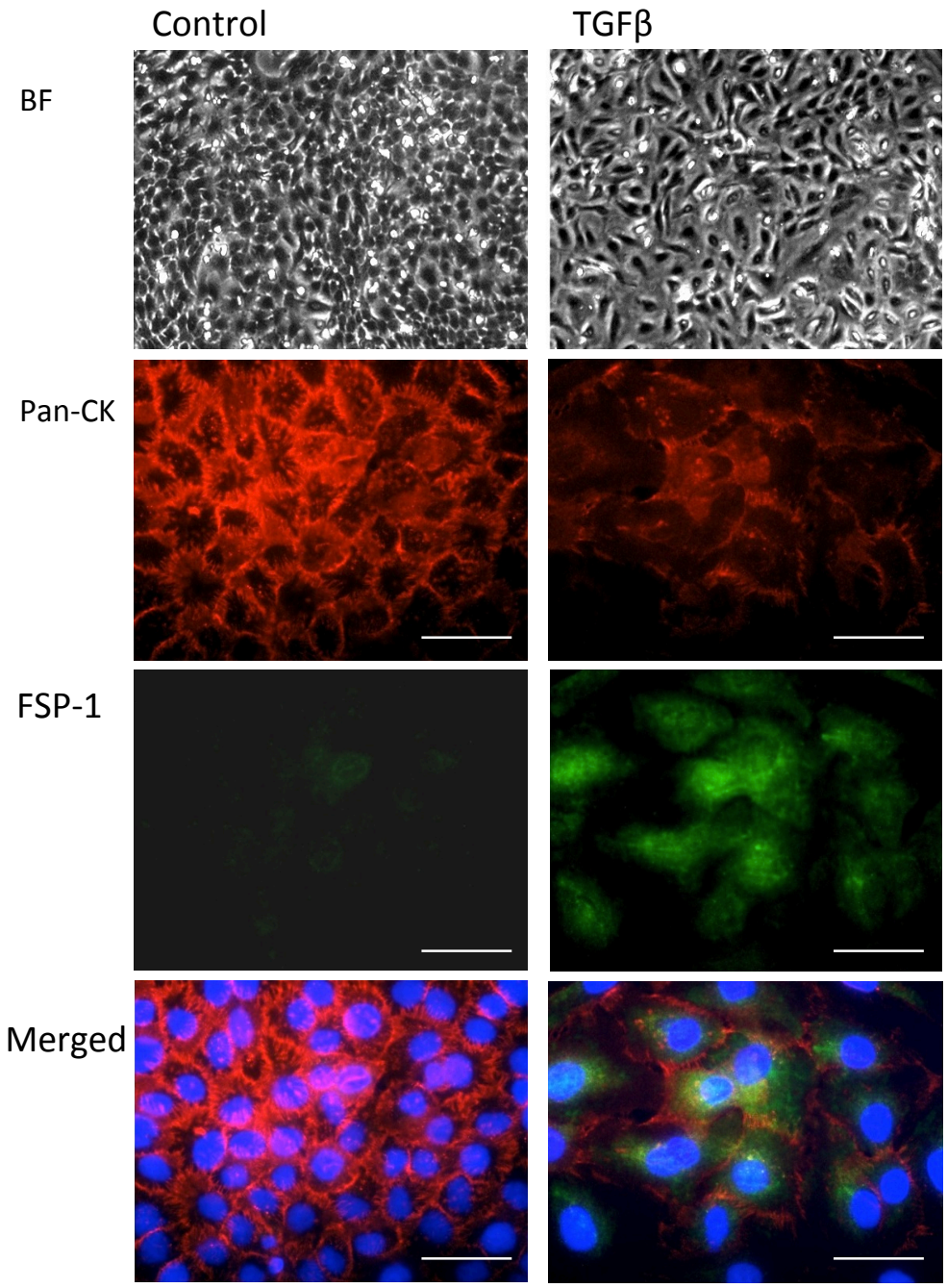


**Figure 5.3. EMT changes in HaCaT cells treated with TGFβ1.**

HaCaT cells were incubated with TGFβ1 for 72hr. Cells morphology was assessed by bright field microscopy (BF), and double immunofluorescent staining was performed to assess effect of the treatment on the expression of epithelial and mesenchymal markers. Pan-cytokeratin (Pan-CK) was used as an epithelial marker (red), FSP-1 was used as mesenchymal marker (green), and DAPI (blue) was used to stain nuclei. The figure shows representative images of 3 independent experiments. Scale bars = 20μm.

However, to confirm these results, Western blotting was performed on lysates of HaCaT cells incubated with 2ng/ml and 4ng/ml of TGFβ1 for 24, 48 and 72hrs. Membranes were probed with α-SMA, E-cadherin, and GAPDH was used as a loading control (Fig 5.4). No differences in α-SMA levels were observed at 24hrs. The highest level of α-SMA was seen in samples incubated with 2ng/ml of TGFβ1 for 48hrs, followed by 4ng/ml of TGFβ1 for 48hrs and 4ng/ml of TGFβ1 for 72hrs. E-cadherin levels did not show any alteration at 24hr, between untreated and samples treated with 2ng/ml of TGFβ1. However, TGFβ1 at 4ng/ml after 24hr started to reduce E-cadherin levels. As expected, incubation with TGFβ for 48 and 72hr lead to decrease in E-cadherin compared to untreated controls.

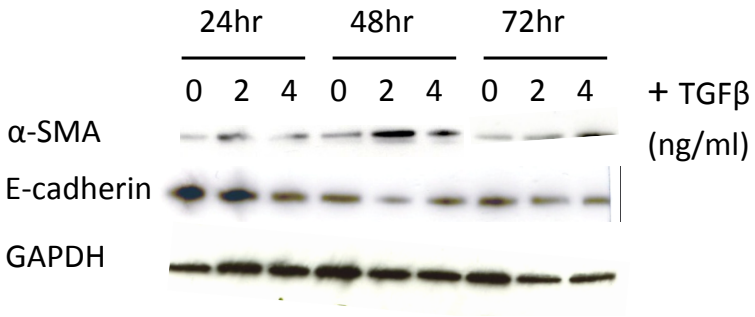


**Figure 5.4. Changes in levels of α-SMA and E-cadherin in HaCaT cells lysates after treatement with TGFβ1.**

HaCaT cells were incubated with TGFβ1 (2ng/ml or 4ng/ml) for 24, 48 and 72hr and lysates used for Western blotting detection of E-cadherin and α-SMA. The image is representative of 3 independent experiments.

Since negative controls samples showed relatively high levels of α-SMA, further studies were performed using FSP-1 as an additional marker of the EMT process, which proved to be more discriminatory as a marker of the mesenchymal phenotype. Consistent with

immunofluorescent results Western blotting analysis of cell lysates showed induction of mesenchymal marker FSP-1 in cells treated with TGFβ1, but not in controls (Fig 5.5).

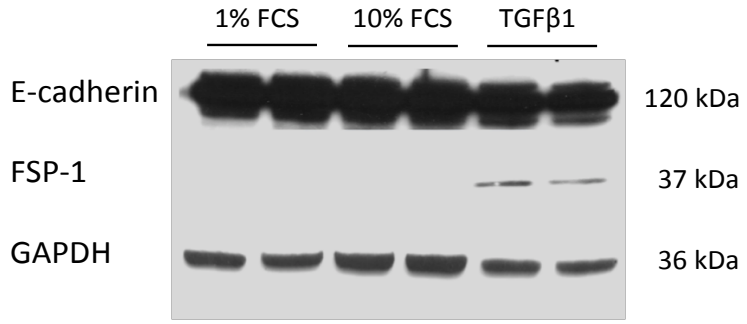


**Figure 5.5. Changes in levels of E-cadherin and FSP-1 in HaCaT cells lysates after treatement with TGFβ1.**

HaCaT cells were incubated with TGFβ1 for 72hr and lysates used for Western blotting detection of E-cadherin, and FSP-1. The image is representative of 3 independent experiments.

Finally, to ensure that the EMT changes can be also detected on the transcriptional level, and to optimize the qPCR assays, RNA was extracted and after RT-qPCR copy numbers of transcription factors involved in EMT were measured. As expected, after 24hrs incubation a 6.9 fold increase in SNAI1 was observed in cells treated with 2ng/ml of TGFβ1 and a 4.3 fold increase in cells treated with 4ng/ml compared to controls (Fig.5.6a). After 48hrs the increase was 6.8 and 2.5 respectively, while after 72hrs the fold increase reduced to 3.6 for 2ng/ml and 3.4 for 4ng/ml treatments.

SNAI2 copy number also increased after TGFβ1 treatment, and after 24hrs incubation was 3.9 times higher in samples where 2ng/ml was used, and 3.4 times higher with 4ng/ml of TGFβ1 when compared to untreated control. However, after 48hrs incubation the fold difference between treated and untreated samples was even higher, 6.7 for 2ng/ml and 4.3 for 4ng/ml. Furthermore, increasing incubation to 72hrs reduced the

difference and samples treated with 2ng/ml exhibited 3.9 fold increase in SNAI2 when compared to untreated, while for 4ng/ml treatment the 2.9 fold increase was recorded.

The results suggest that the optimal concentration of TGFβ1 for EMT induction in HaCaT cells is 2ng/ml for 24-48hrs, and the best time point for SNAI1 mRNA detection is 24-48hrs but for SNAI2 it is 48hrs.

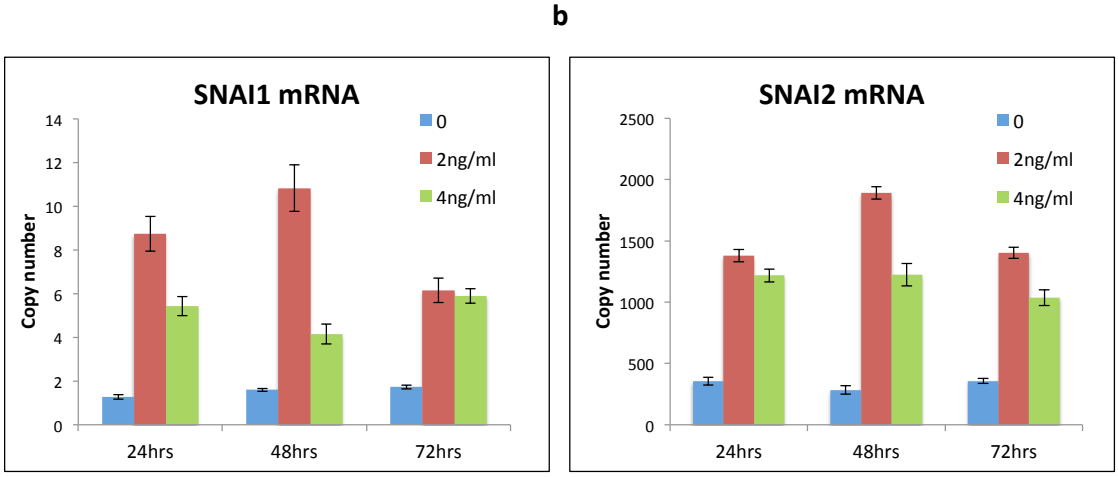


**Figure 5.6. Changes in mRNA levels of Snail1 and Snail2 in HaCaT cells lysates after treatement with TGFβ1.**

HaCaT cells were incubated with TGFβ1 for 24- 72hr and cell lysates used for RT-qPCR detection of SNAI1 and SNAI2. The image is representative of 3 independent experiments. The graphs show mean ± SEM.

### 5.2.3 Continuity of the basement membrane in SSc skin

Once assured that potential EMT changes will be detected by the optimized assays, analysis of patient's samples was initiated. Paraffin embedded sections of forearm skin of healthy controls and SSc patients were stained using antibodies against mesenchymal marker FSP-1 and the basement membrane protein – collagen IV. The double immunofluorescent staining results showed FSP-1 staining in control tissue around blood vessels and single positive cells in the basal epidermal layers and dermis (Fig.5.7).

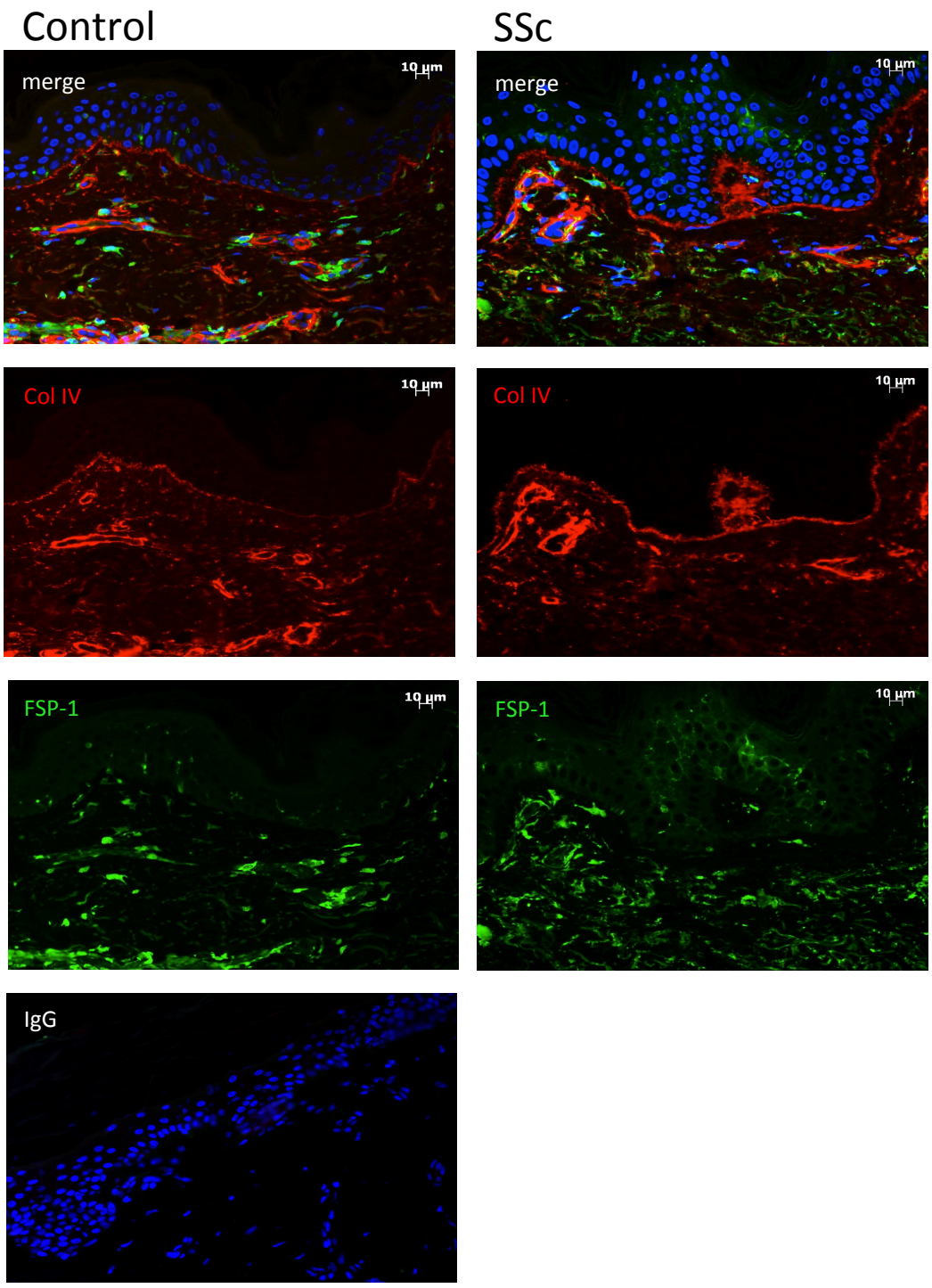


**Figure 5.7. Immunofluorescent staining of collagen IV and FSP-1 in SSc skin.**

Representative images of double immunofluorescent staining performed to detect basement membrane protein collagen IV (red) and mesenchymal cell marker FSP-1 (green) in the epidermis of SSc patients and controls forearm skin sections. DAPI (blue) was used to stain nuclei.

FSP-1 staining was increased in SSc, where not only more positive cells were observed in the epidermis, but in contrast to basal location in controls they were present also in the suprabasal layers. In the SSc dermis FSP-1 signal was detected around blood vessels, as well as bound to ECM. However, collagen IV in the basement membrane of SSc patients did not appear compromised and the thickness of the basement membrane did not show any changes. As expected, staining was restricted to the epidermal-dermal junction and areas around blood vessels. There were also no indications of cells that were migrating through the membrane from the epidermis into the dermis.

### 5.2.4 Double immunofluorescence for vimentin and E-cadherin

Despite lack of evidence for compromise to or migration of cells through the basement membrane we decided to investigate further if any signs of EMT are present in the SSc skin. Unfortunately, there is no single specific marker available that would allow distinctive recognition of fibroblasts from other types of cells of mesenchymal origin. Therefore, I have chosen on this occasion to use vimentin, as a mesenchymal marker and looked for cells that would co-express it with E-cadherin to indicate EMT.

Analysis of double staining of vimentin with E-cadherin, did not reveal any differences in pattern or level of E-cadherin expression, between control and SSc skin sections (Fig.5.8). Vimentin staining was very intense in the dermis of control and SSc. Interestingly, similar to FSP-1 staining results; positive cells were detected in the epidermis, with basal location in the controls and suprabasal in the SSc. Moreover, those cells were positive for both vimentin and E-cadherin. As already mentioned in the introduction chapter such co-expression of epithelial and mesenchymal markers could indicate EMT process or else be due to local dendritic cells, which can express both markers.

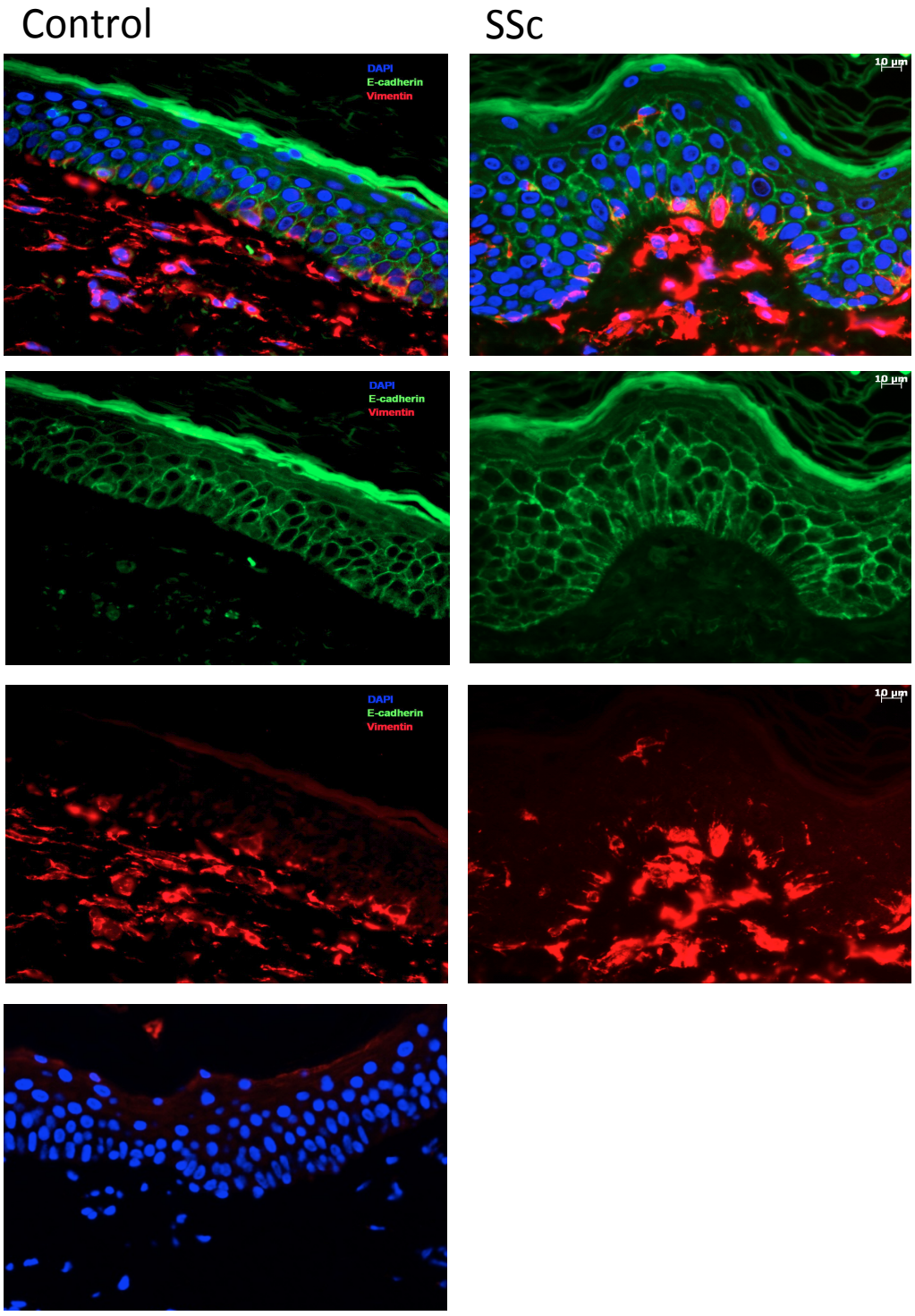


**Figure 5.8. Immunofluorescent staining of E-cadherin and vimentin in SSc skin.**

Representative images of double immunofluorescent staining performed to detect epithelial cell marker E-cadherin (green) and mesenchymal marker vimentin (red) in the epidermis of SSc patients and controls forearm skin sections. DAPI (blue) was used to stain nuclei.

### 5.2.5 Langerin and FSP-1 co-staining

An epidermal population of dendritic cells called Langerhans cells (LCs) contributes 3-5% of nucleated cells in the epidermis (Merad et al., 2008). LCs express E-cadherin to attach to keratinocytes (Valladeau and Saeland, 2005), and due to their hematopoietic origin (Stingl et al., 1980) also express vimentin (de Waal et al., 1984) and FSP-1 (Boni et al., 1997). To establish if LCs are the source of vimentin and FSP-1 positive cells in the epidermis and therefore confounding the results above, langerin (CD207) a type II trans-membrane lectin receptor on LCs was used along with FSP-1.

The results of immunofluorescent staining of skin sections revealed that all cells positive for FSP-1 were also positive for langerin. This suggests, that those cells belong to a subset of dendritic cells expressing langerin (most likely LCs), rather than cells undergoing EMT.

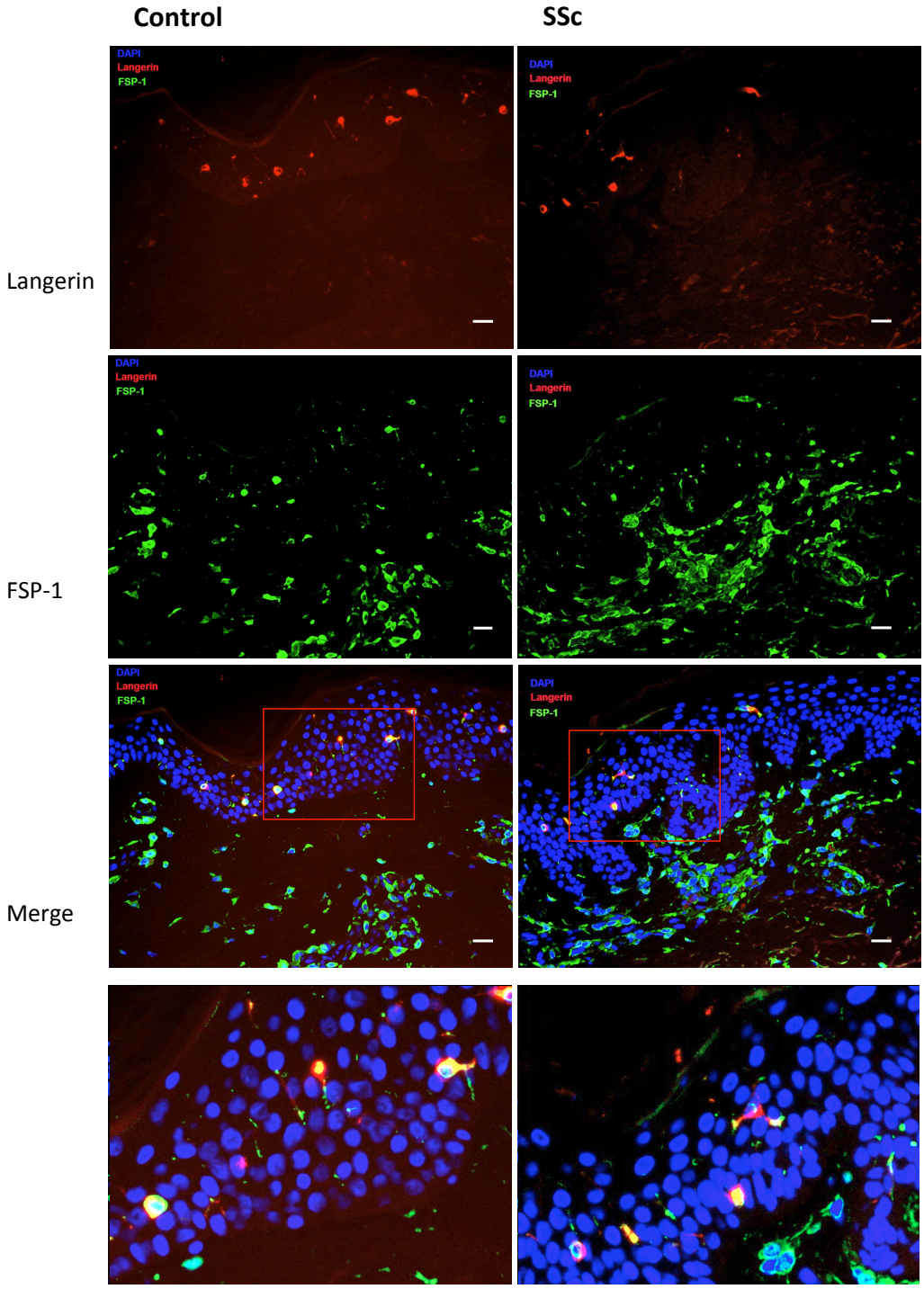


**Figure 5.9. Co-staining of langerin and FSP-1 in control and SSc epidermis.**

Representative images of double immunofluorescent staining performed to detect Langerhans cell using langerin (red) and mesenchymal cells using FSP-1 (green) in the epidermis of SSc patients and controls forearm skin sections. DAPI (blue) was used to stain nuclei.

### 5.2.6 Fibrotic markers in the SSc skin

As demonstrated in previous sections of this chapter the SSc skin shows an increased staining of mesenchymal markers, vimentin and FSP-1. Although, fibrotic markers such as α-SMA and pro-collagen I were extensively studied in the dermis, the epidermal compartment of the skin was often omitted in the published reports as irrelevant to the disease process. In order to examine the expression of these markers in the SSc epidermis, α-SMA and pro-collagen type I immunohistochemistry was performed on paraffin embedded skin sections.

Consistent with a previous study (Farina et al., 2009), staining with α-SMA antibody (Fig. 5.10) revealed a higher number of positive cells, especially in the SSc upper dermis in the proximity of the epidermis when compared to healthy skin. Moreover, 43% of SSc sections (3 out of 7) had positive α-SMA staining in cytoplasm of epidermal keratinocytes of basal and suprabasal layers compared to 0 of 6 controls. All of the sections with the epidermis positive for α-SMA came from late stage dcSSc patients.

Cells positive for pro-collagen type I were found in both healthy and SSc dermis (Fig.5.11A). However, in contrast to control and early SSc sections, the extracellular staining present especially in the upper dermis was absent in 2/3 of late SSc samples. At the same time, the number of dermal cells expressing pro-collagen in the late SSc increased from 18.5±3.9 in controls and 19.3±5.7 in early stage disease to 40.3±4.3 (both control vs. late stage disease and early vs. late stage disease $p<0.05$), illustrating the amplified number of myofibroblasts with disease progression (Fig.5.11.B). The different pattern of pro-collagen expression between different stages of disease suggests that, as SSc advances there might be a change in the epidermal-dermal cross talk, as the extracellular pro-collagen was mainly detected in the papillary dermis in early stages of the disease. Such loss of extracellular pro-collagen type I points also at the alteration in components of the ECM in the SSc.

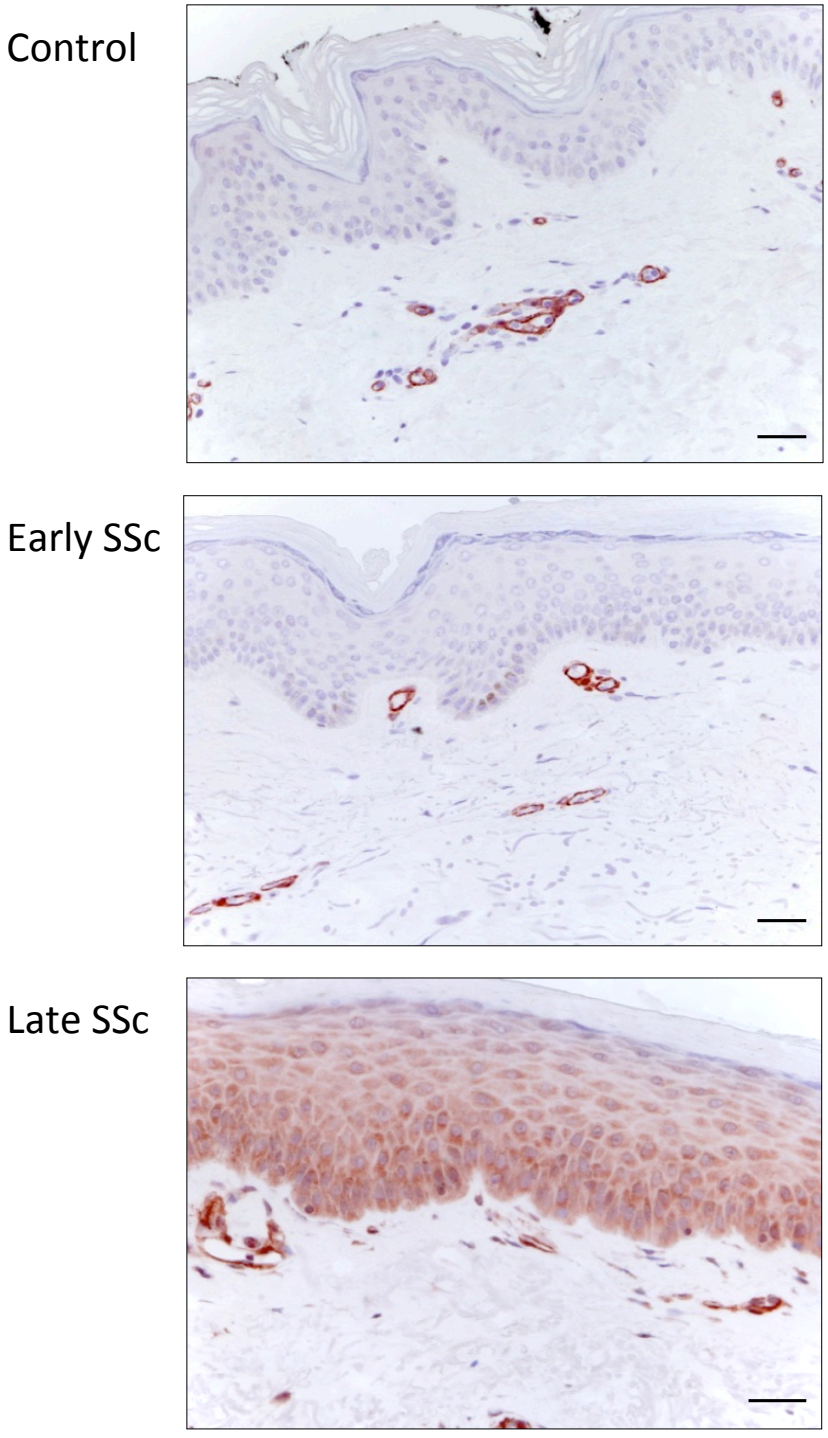


**Figure 5.10. Elevated levels of α-SMA in epidermis of late SSc patients.**

Representative images of forearm skin sections of patients with early and late SSc, and controls stained with α-SMA.

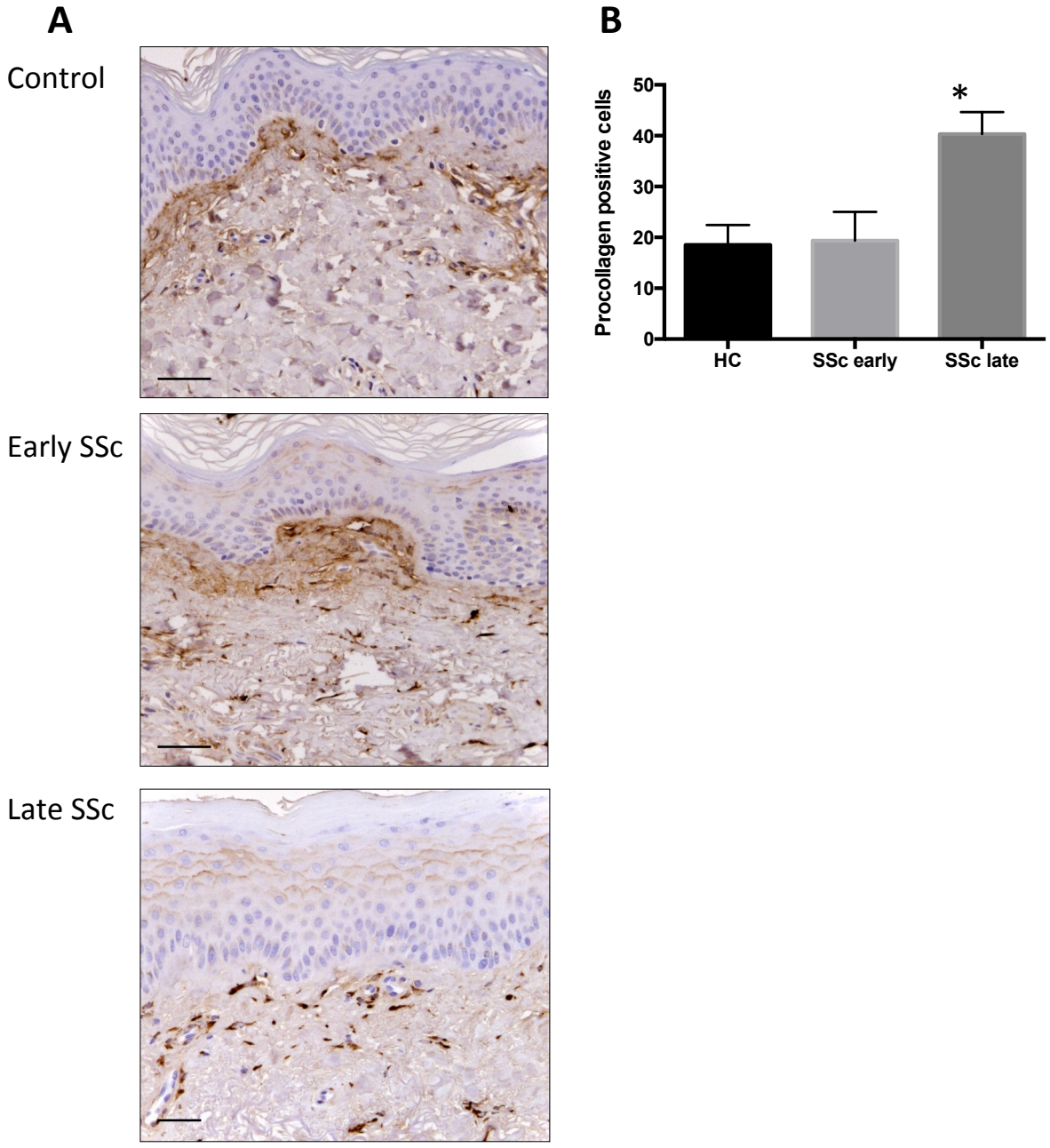


**Figure 5.11. Increased levels of intracellular type I with decrease in extracellular levels in the papillary dermis of late SSc patients.**

**(a)**Representative images of forearm skin sections of patients with early and late SSc, and controls stained with pro-collagen type I. Cells showing positive staining were counted and mean was calculated for each section based on average of 3 high magnification fields of view. **(b)** Mean± SEM of type I pro-collagen positive cells in the dermis of control subjects, early and late SSc patients (n=4, n=3 and n=3 respectively). The one-way ANOVA followed by Tukey's multiple comparison test was used to calculate statistical significance, * $p< 0.05$.

The interesting results of diminished extracellular bound type I pro-collagen at the epidermal dermal junction in late SSc was followed by assessment of pro-collagen mRNA in the epidermal blisters. The gene encoding alpha1 chain of type 1 pro-collagen (*COL1A1*) was measured in extracts from epidermal tissue samples and showed a significant reduction in type I collagen mRNA levels with disease progression in the epidermis of SSc patients ($p<0.05$). The late SSc patients' epidermis contained 2.2 times less pro-collagen mRNA (56.5±7.98) when compared with control (122.2±26.54) and 1.7 fold reduction than in early samples (96±19.56) (Fig.5.12). There was no significant difference in the relative copy number of this gene between controls and early SSc patients. Such correlation between epidermal mRNA and extracellular levels of pro-collagen at the epidermal-dermal interface further strengthen the interplay between these two skin components.

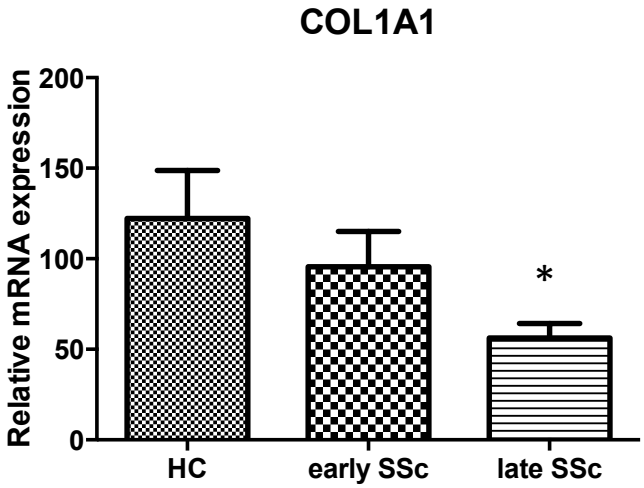


**Figure 5.12. Decreased pro-collagen type I mRNA in the epidermis of late SSc patients.**

Levels of *COL1A1* mRNA in epidermal blister sheets were compared between control, early and late dcSSc disease. HC - control (n=6), early SSc – early diffuse SSc (n=3), late SSc – late diffuse SSc (n=8). The one-way ANOVA followed by Tukey's multiple comparison test was used to calculate statistical significance, * $p< 0.05$.

### 5.2.7 mRNA assays for EMT markers in epidermal blister sheets

To ensure that the investigation of possible EMT evidence in SSc epidermis is carried out also at the mRNA level, qPCR assays were included for key markers of EMT, Snail1 and Snail2. Samples were normalised using normalisation factor calculated by GeNorm software based on expression of most stable genes TUBB and B2M.

Analysis of blister epithelial sheets revealed a 2.4 fold increase in number of *SNAI1* copies ($p<0.05$) in SSc compared to controls, which among healthy subjects included patients with primary Raynaud's syndrome. However, no significant differences were observed with *SNAI2* between control and SSc samples.

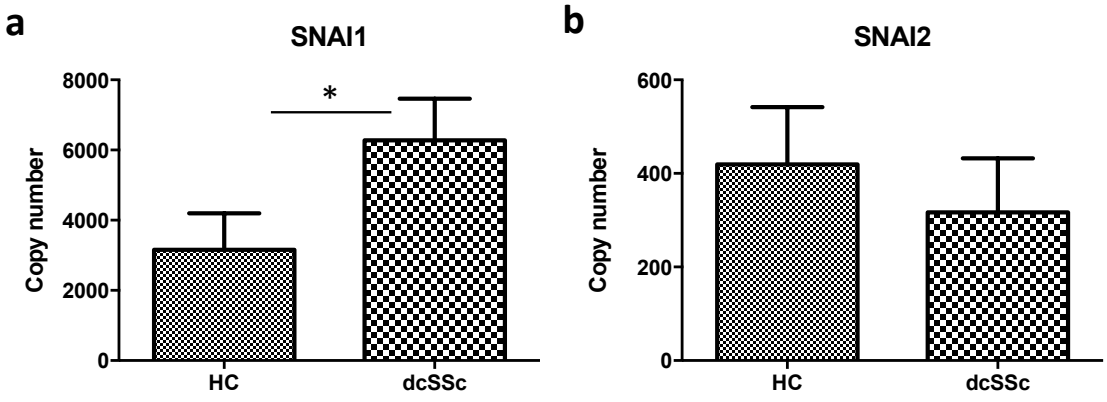


**Figure 5.13. SNAI1 and SNAI2 mRNA in epidermal blister sheets.**

Copy numbers of **(a)** *SNAI1* and **(b)** *SNAI2* mRNA were measured in the epidermal blister sheets of SSc patients and controls. The results are presented as mean ± SEM. The Mann-Whitney U test was used to calculate statistical significance, * $p<0.05$. HC- control (n=9), dcSSc – diffuse SSc (n=14).

## 5.3 Discussion

Chapter 4 has demonstrated elevated levels of pro-inflammatory S100A9 and pro-fibrotic CTGF in SSc epidermis. Both proteins have been recently shown to be associated with the EMT process (Sonnylal et al., 2013, Cormier et al., 2014). Therefore in this chapter, the effect of the inflammatory microenvironment and active TGFβ signalling on potential EMT changes in SSc skin has been examined. The presence of active Smad signalling in the SSc epidermis and papillary dermis has been clearly demonstrated. In addition possible expression of markers of the EMT process, has been investigated it the SSc epidermis. Although, an *in vitro* model of EMT in HaCaT positively identified changes consistent with the transition of keratinocytes, no definite answers were found in SSc skin. This work complements work carried out on EMT in SSc skin by Gillespie and Nakamura (Gillespie et al., 2011, Nakamura and Tokura, 2011).

As described in previous reports (Rasanen and Vaheri, 2010, Lamouille and Derynck, 2007), I found that TGFβ1 treated cells begin to change morphology to more elongated, as seen under bright field microscope. Immunohistochemistry of HaCaT cells incubated with TGF-β showed an increase in FSP-1 expression coupled with decreased Pan-cytokeratin when compared with controls, which was expected and indicates a switch from epithelial to mesenchymal markers. The result was confirmed by Western blotting, where TGFβ1 treatment led to down-regulation of E-cadherin, consistent with Wang's data (Wang et al., 2012), and upregulation of FSP-1 protein. Moreover, cells incubated with TGF-β had significantly raised levels of SNAIL1 and SNAIL2 mRNA when compared with untreated, consistent with previous report (Herfs et al., 2008). The time course confirmed that both SNAIL1 and SNAIL2 are induced early on during the EMT, and precede any changes to protein markers. All these observed changes suggest that the cells treated with TGFβ were undergoing EMT. The finding is consistent with previous reports of TGFβ stimulation of epithelial cells to undergo EMT reviewed by Zavadil (Zavadil and Bottinger, 2005).

Hypothetically, a portion of fibroblasts observed in SSc skin could originate from proliferation of local fibroblasts, bone marrow derived fibroblasts progenitors or epithelial/endothelial derived fibroblasts via EMT and EndMT. Studies on other organs have indicated the potential importance of EMT in SSc. Moreover, I had found that upregulated TGF-β signalling via Smad2/3, known to promote EMT, and activated keratinocytes, are both features of SSc skin. Thus, this work is an attempt to address a question: is there any evidence for EMT in SSc skin?

During the course of my study reports suggesting EMT in SSc skin started to appear. Gillespie (Gillespie et al., 2011) described basal cells of the SSc epidermis with increased SFRP4, lack of Caveolin-1 and decreased E-cadherin expression along with the expression of vimentin. These findings suggest that due to inhibition of WNT signalling basal cells undergo EMT and thus are directly involved in SSc pathogenesis but was reported only in a conference abstract form, making the results are more difficult to scrutinize. Another association between EMT and SSc skin was brought by Nakamura, who reported that Twist and Snail1 positive cells were found within eccrine glands of SSc patients (Nakamura and Tokura, 2011). Although interesting, the report was based on a limited number of SSc samples, and therefore might not be truly representative to the wider cohort of SSc patients.

To obtain more evidence of the occurrence of EMT in SSc skin, and gain more insight into its role in SSc skin pathogenesis a series of experiments were set up to detect EMT changes in SSc skin sections and epidermal blisters. As anticipated, immunostaining revealed activated canonical TGF-β signalling in SSc epidermis illustrated by nuclear staining for phosphorylated Smad2/3. Additionally a rise in Smad2/3 phosphorylation was observed in the dermis adjusted to the epidermis, confirming cross talk between the activated epidermis and dermis. However, despite enhanced TGF-β signalling no loss of E-cadherin was observed in epidermal keratinocytes of SSc patients. In both healthy control and diseased epidermis some cells positive for vimentin and FSP-1 were observed, although, they co-stained for langerin, indicating that these cells are

Langerhan's cells rather than epidermal keratinocytes undergoing EMT. In SSc skin sections enhanced expression of the FSP-1 marker in the dermis was observed which was expected due to the higher number of myofibroblasts in the fibrotic dermis.

Likewise, no decrease of collagen IV was observed in SSc sections that would suggest degradation of the basement membrane, which would enable cells undergoing EMT to migrate into the dermis. Interestingly, some of the sections stained with α-SMA antibody gave positive results in the late SSc epidermis. Detection of this mesenchymal marker often induced during EMT in SSc epidermis was unexpected, but repeated a number of times the staining looks specific and gave consistent results. However, to definitely confirm the specificity of the staining blocking peptide should be used. The presence of α-SMA positive cells in SSc epidermis may suggest some partially evolved EMT process, or at least an altered program of differentiation in the SSc skin.

Production of collagen type I have been shown to be upregulated in dermal fibroblast in SSc by a number of studies (LeRoy, 1974, Krieg et al., 1978). The protein is synthesized in a precursor form as a pro-collagen, which is converted into collagen by enzymatic cleavage of amino-and carboxyterminal peptides. These pro-collagen peptides are used as markers of collagen biosynthesis, but are also involved in a regulatory process of collagen production (Krieg et al., 1978, Fouser et al., 1991, Paglia et al., 1979, Horlein et al., 1981). Especially, the amino-terminal pro-collagen peptides were demonstrated to significantly reduce collagen production by fibroblasts via negative feedback loop, and decreased levels of these peptides in SSc were shown to contribute to disregulated collagen production by SSc fibroblasts (Krieg et al., 1978).

In this chapter pro-collagen type I expression pattern was observed to change with disease duration. In SSc patients with early stage of disease pro-collagen type I was mainly located bound to the ECM at the epidermal dermal junction, comparable to controls. However, with disease progression the extracellular expression in the papillary dermis decreased, while the number of cells in the papillary dermis expressing pro-

collagen type I increased. This is in contrast with a reported 3 fold increase in concentration of C-terminal peptide in serum of dcSSc patients when compared to control (Kikuchi et al., 1994). These opposite results may be due to the fact that the antibody I have used for detection of type I pro-collagen in skin sections binds to the N terminal of pro-collagen, which is degraded more easily than the C-terminal peptide which circulates in blood. However, this does not explain the differences in pro-collagen expression in different stages of the disease.

This change in pro-collagen expression suggests altered collagen type I processing in established SSc. Perhaps pro-collagen cleavage in established SSc is as some suggest intracellular (Bienkowski et al., 1978, Kadler et al., 2007) while in controls and early disease the cleavage is at the membrane in fibropositors. Moreover it is possible that generation of collagen fibrils occurs faster in the patients with late SSc, therefore the N-terminal peptide in late SSc is seen only inside the cells and not in the ECM. However, described in this chapter reduction in N-terminal pro-peptide at the epidermal-dermal junction, could also be linked to the overexpression of collagen in the SSc, due to lack of negative feedback loop (Krieg et al., 1978).

Although the pro-collagen mRNA was previously shown to be raised in SSc skin fibroblasts (Graves et al., 1983), the results of mRNA analysis of gene encoding pro-α2(I) chain of type 1 collagen performed on epidermal tissue shows lower levels in late SSc than in controls. Detection of this procollagen in the epidermal sheets rises questions regarding specificity of the primers used, as type I collagen is not normally present in the epidermal cells. Indeed *COL1A1* shows some degree of homology with other procollagens. However, presence of pro-α2(I) chain of type 1 collagen in the mRNA derived from epidermal sheets but may also indicate dermal contamination of the samples. However, if the finding was real this would further point to altered collagen processing with the disease progression.

To gain a more definite answer regarding the possible EMT in SSc, staining for Snail1 and Snail2 was performed on skin sections, but despite trying different antibodies and

optimizing protocols the staining was very unspecific (not shown). Therefore, the levels were analysed in RNA extracted from epidermal blister sheets, showing doubling of *SNAI1* mRNA copy number in SSc compared to controls. Such an increase in gene expression, encoding an EMT-inducing transcription factor may suggest an active EMT process in the SSc epidermis. Although no significant change in *SNAI2* was observed, this gene is usually expressed later on during transition process. Thus absence of *SNAI2* induction in SSc epidermis does not have to indicate absence of EMT but may imply early stage of these changes.

Although, active Smad/TGF-β signaling, presence of αSMA in epidermis and enhanced CTGF expression, along with enhanced levels of *SNAI1* mRNA in SSc suggest EMT changes, other results obtained so far do not indicate EMT changes in SSc skin. Therefore, SSc epidermis might be a scene of partial EMT that has already been reported during wound re-epithelialisation (Arnoux et al., 2008, Yan et al., 2010, Savagner et al., 2005) Considering that the SSc keratinocytes phenotype is similar to that seen in wound healing this explanation might be probable. However, substantially more work should be done to get a definitive answer, preferably lineage tracking of labeled epidermal cells and therefore would require an animal model.

# Chapter Six

# Summary Discussion and Future Work

This thesis was set out to explore the concept of the SSc epidermis playing an important role in propagation of skin fibrosis by releasing pro-inflammatory and profibrotic mediators stimulating underlying fibroblasts. In this thesis proteins involved in SSc dermal fibroblast stimulation have been identified, as well as pathways and biological processes that are altered in the SSc epidermis, which can contribute to the disease. Also I have confirmed that keratinocytes are activated in SSc skin, and have altered differentiation and proliferation processes. In addition I have sought to investigate evidence of EMT changes in epidermal keratinocytes of SSc patients. Although no signs of the complete EMT process was found, I showed changes consistent with a partially evoked EMT.

The background knowledge available on this subject has been limited and inconclusive. My findings however clarify the importance of the epidermis in the context of understanding SSc pathogenesis. This chapter aims to summarise my novel findings, which have been presented in the results chapters addressing the main research questions asked in the thesis, but also discusses the contribution to the field, limitations of the studies, as well as future works that could further explore the areas studied in this thesis.

## 6.1 Epidermal hypertrophy and premature expression of differentiation markers in SSc

I was able to demonstrate significant increase in the epidermal thickness in SSc skin, which was consistent with more recent reports (Van Praet et al., 2011, Rossi et al., 2010), but contradicted older observation describing SSc epidermal atrophy (Cooper et al., 1979). This dissimilarity between results from older and recent publications could be caused by different classification of SSc disease, which has changed considerably over the years and was not clearly defined till the 80s (LeRoy et al., 1988). I have also shown that this thickening of the SSc epidermis could be linked to abnormal differentiation of SSc keratinocytes, as well as hypertrophy of SSc keratinocytes, and increased proliferation of epidermal cells in the disease. The parakeratosis, which I found to be

present in some of the SSc sections, was consistent with previous reports (Van Praet et al., 2011, Maeda et al., 1993). Changes in the SSc epidermal keratinocyte differentiation program based on the expression pattern of keratins K14, K1/10 were also previously reported (Aden et al., 2008), however the markers of differentiation used in this thesis were never studied previously in the SSc skin.

Premature expression of involucrin, loricrin and filaggrin described in this thesis is characteristic to hyper-proliferative keratinocytes of wounds and psoriasis skin (Hertle et al., 1992, Li et al., 2000, Mansbridge and Knapp, 1987), and further strengthens evidence of similarities between SSc epidermis and wound re-epithelialisation. This is in line with results described in this thesis revealing a trend towards increased proliferation of SSc keratinocytes and consistent with previous findings (Pablos et al., 1999). However, my proliferation analysis had included only limited number of samples studied. Increasing the number of samples, closer age matching of controls and patients and stratifying patients with early and established disease, plus taking into consideration mRSS of patients, would strengthen the resolving power of this analysis. It would also be interesting to look at the behavior of SSc keratinocytes in culture, to find out if they would retain their phenotype. Also studying their behavior during calcium switching experiments could provide some detail on the triggers and mechanisms of such abnormal differentiation.

## 6.2 Protein phosphorylation in the SSc epidermis

This thesis demonstrates that not only the morphology of the SSc epidermis is affected but also phosphorylation and abundance of the epidermal proteins. Proteins with altered levels of phosphorylation in the SSc epidermis include phosphatases, kinases, and proteins associated with the SSc pathogenesis, such as Smad2 and PDGFR. Overall I report extensive changes in phosphorylation status in diverse proteins involved in cell motility, polarity and chemotaxis, apoptosis, cell adhesion, cell cycle regulation, as well as cytoskeletal reorganization, differentiation, and regulation of transcription. The results

presented in this thesis indicate that SSc epidermal cells have an activated response to stress and wound healing, increased activation of innate and adaptive immune system, signal transduction, pathways implicated in platelet activation and blood coagulation. Therefore my findings are consistent with the general knowledge and current model of processes contributing to fibrosis and inflammatory changes seen in SSc.

The main limitation of this study was the small number of samples used in the assay. Moreover the epidermis was separated from the dermis using a scalpel after snap freezing, which could result in dermal contamination. Therefore if repeated in the future it would be useful to consider other rapid but more accurate methods of separation. It would also be interesting to see if the altered phosphorylation is linked to alternative splicing or mutation, for example the reduction of phosphorylation at a particular site might be due to the splice variant/mutated gene that results in the protein that lacks that phosphor site. Also selecting a protein with a changed phosphorylation status, and studying in detail the functional effect of the change *in vitro* in the context of fibrosis and inflammation could complement work presented in this thesis.

### 6.3 Epidermal secretome

As shown by my work, SSc epidermal biopsies release a wide range of cytokines, chemokines, and growth factors into the media, thus demonstrating the potential importance of the epidermis as a secretory organ able to influence behavior of cells including underlying fibroblasts and endothelial cells, as well as cells of the immune system.

The main limitations of the skin explant proteome analysis were wide variations in the normal control and disease concentration values. This could be limited in the future by recruitment of more closely matched controls, as well as obtaining larger numbers of skin biopsies, although this is challenging in an uncommon condition and using a relatively invasive sampling method. Moreover, including more acute untreated cases, and

stratification of patients into early and late SSc would help to reduce variability between samples, and reduce standard deviation of the mean. In addition, if the experiment could be repeated in the future, the volume of the media should be reduced to increase concentration of the soluble factors and therefore avoid problems with detection. Moreover, performing Western blot analysis of the explants after collecting the media to compare levels of proteins released into the media with levels of ECM or cell-bound, would help in better understanding of the abnormalities seen, including signaling pathways and their downstream targets.

### 6.3.1 S100A9

Immunofluorescent staining of skin sections, as well as epidermal and dermal explant secretome analysis presented in this thesis have demonstrated that S100A9 is overexpressed by SSc keratinocytes, and released in higher concentrations by SSc epidermis than controls. Results of the epidermal blisters sheet analysis also show a trend towards an increase in S100A9 mRNA. Although S100A9 was previously reported to be increased in SSc serum, feces and saliva of SSc patients (Andreasson et al., 2011, Giusti et al., 2007), only recently and synchronous to work on this thesis upregulated levels have been reported in the SSc skin (Xu et al., 2013b). Nonetheless this paper does not show pictures of stained epidermis, and only discusses of S100A9 expression pattern in the dermis. Therefore, the data on S100A9 expression in the epidermis and the keratinocytes as the main sources of this protein in the SSc skin are novel observations. Also in contrast to previous observations of S100A9 being overexpressed along with its heterodimer partner S100A8 in the SSc dermis and plasma, on both protein and mRNA levels (Xu et al., 2013b), staining and mRNA analysis presented in this thesis does not confirm that in SSc epidermis S100A9 forms heterodimer. Furthermore, my thesis shows additional novel data revealing that S100A9 stimulates proliferation and migration of SSc dermal fibroblasts, induces CTGF protein and mRNA in cultured dermal fibroblasts from both SSc and control, as well as enhances IL-6 expression in HaCaT cells. In addition, I have

now shown that these effects are mediated via TLR4 because I found that TAK-242 an inhibitor of TLR4, blocks the effects of S100A9 on CTGF protein and mRNA.

Overall my data suggests a link between pro-inflammatory and pro-fibrotic pathways that may play a role in the SSc pathogenesis (Nikitorowicz-Buniak et al., 2014). It also emphasizes the potential for epithelial damage or activation to modulate dermal fibroblast properties by induction of key growth factors, and demonstrates the importance of interactions between epidermal keratinocytes and dermal fibroblasts. My work also adds to the field of knowledge implicating S100A9 in fibrotic conditions, for example the potential to activate lung fibroblast via RAGE (Xu et al., 2013a). Furthermore, considering associations of elevated S100A9 plasma levels with clinical observations such as pulmonary fibrosis, kidney involvement and myositis (Xu et al., 2013b), the finding of SSc keratinocytes as a source of this protein is consistent with an important role for the epidermis in SSc pathogenesis.

My findings may have some therapeutic application as pharmacological compounds such as quinoline-3-carboxamide (Q compound) have recently been found to bind S100A9, limiting its downstream signalling (Bjork et al., 2009). This drug is currently in clinical trials for treatment of metastatic prostate cancer (Pili et al., 2011, Dalrymple et al., 2012). As currently there is no satisfactory therapy for SSc our findings suggest that Q compound or other drugs targeting S100A9 might have some beneficial effect, which could be investigated further in SSc.

The analysis of S100A9's impact on fibroblast and keratinocytes however has a few restraints, which should be considered. S100A9 is a Ca2+ binding protein and the effects on cellular functions ideally should be performed after optimizing Ca2+ concentration. Moreover, to fully verify the impact of stimulation with S100A9 on fibroblasts, the recombinant S100A9 should be used in combination with other factors that are upregulated in SSc skin, such as TGFβ1 or CTGF. This would more truly reflect the disease microenvironment that cells are exposed to in SSc tissues. It is possible that such combinations could have an additive effect to the S100A9 itself. Also autocrine effects of

S100A9 overexpression by SSc keratinocytes would be more effectively studied by creating a keratinocyte line overexpressing the gene. This could be achieved by using keratinocytes from a S100A9 overexpressing transgenic animal strain, or transfecting keratinocytes with *S100A9* gene using a viral or non-viral transfection agent. However, keratinocytes due to constant exposure to viruses have developed mechanisms preventing hijacking by viruses, and thus are relatively difficult to transfect. The efficiency of transfection is usually low and often leads to induction of terminal differentiation, thus any attempts to generate the transfected line were beyond the timescale limits of this thesis.

If I were not bound by the time constraints of this thesis, I would also look at S100A9 signalling via RAGE in the SSc skin. Moreover, I would study cutaneous fibrosis and inflammation in transgenic mice overexpressing S100A9 under the K14 promoter, to ensure overexpression only in epithelial cells, treated with bleomycin to induce fibrosis. Such approach would not only be a better model for studying the importance of S100A9 on different cell types relevant to SSc pathogenesis in their biological niches, but would also allow to look at any changes in interactions between keratinocytes, fibroblasts, endothelial and immune cells, for example leukocyte migration. Therefore, this would allow more comprehensive understanding of the relevance of S100A9 in SSc skin disease.

### 6.3.2 CTGF

Although CTGF overexpression was previously reported to be present in the skin of SSc patients (Igarashi et al., 1996) the report did not include comment or analysis of the diseased epidermis or keratinocytes. This thesis demonstrates not only enhanced levels of CTGF protein and mRNA in the SSc epidermis, but also shows increased release of CTGF by the diseased epidermis. The contribution of CTGF to the fibrosis in SSc is well established and its plasma and dermal interstitial fluid levels correlate with severity of skin disease (Dziadzio et al., 2005). Therefore, production of this key factor driving

fibrosis in SSc by the SSc epidermis indicates a direct contribution of the epidermis to the SSc pathogenesis.

Moreover, a pronounced CTGF expression, detected at the epithelial/dermal junction especially in early stages of disease, suggests involvement of epidermal cells in stimulation of dermal production of CTGF. These detected differences in the CTGF expression could help to explain the higher serum levels in early SSc previously reported (Sato et al., 2000). Additionally, the increase of CTGF in early SSc samples coincides with higher levels of S100A9 in the epidermis, and as shown in this thesis the S100A9 stimulation of dermal fibroblasts also results in the CTGF production. It would be interesting to recruit early and late SSc patients and determine whether the levels of S100A9, and CTGF in plasma and skin correlate. Such observation would allow to further understand the observed *in vitro* S100A9 mediated CTGF expression.

The low levels of CTGF detected in the dermal explants conditioned media were not anticipated. However this could be caused by the CTGF being bound to ECM matrix or receptors and thus not as freely released as that by epidermal explants. Thus it could be useful to perform co-staining of the sections derived from the explants with CTGF and its most relevant receptors. Alternatively, co-immunoprecipitation could be performed on the explant tissue.

### 6.3.3 HGF

The secretome analysis presented in this thesis has shown an increased release of HGF from the SSc epidermis and dermis when compared with controls. Although the concentration of HGF was higher in the SSc epidermis than SSc dermis, immunostaining and mRNA assay results suggest that keratinocytes are a target rather than the main source of HGF in the SSc skin. This is consistent with the previous work showing increased c-Met signaling in the SSc epidermis (Aden et al., 2010). Increased presence of

this anti-differentiation factor could be implicated in delayed differentiation of SSc keratinocytes.

Previous works showing an enhanced HGF mRNA levels in SSc skin (Frost et al., 2012), and amplified expression in SSc fibroblasts along with its receptor c-Met (Kawaguchi et al., 2002) also point at the SSc dermal fibroblasts as the main source of this growth factor in the SSc skin. Therefore, again it would be useful to compare levels in the media with the levels bound to c-Met receptor in both epidermal and dermal explants. Nevertheless enhanced levels of HGF demonstrated in this thesis, might be a form of compensatory mechanism to the fibrotic changes due to its ability to suppress collagen synthesis and CTGF in SSc fibroblasts (Bogatkevich et al., 2007, Jinnin et al., 2005, Sherriff-Tadano et al., 2006).

### 6.4 TGFβ/Smad signaling and EMT

In this thesis I demonstrated upregulated TGF-β signalling via Smad2/3 in the SSc epidermis and adjacent dermis. The active canonical TGF-β signaling in SSc skin is consistent with previous study showing nuclear location of phosphorylated Sman2/3 in the SSc skin including the epidermis (Dong et al., 2002). TGF-β signaling is known to promote EMT, and since activated keratinocytes are seen in SSc skin hypothetically, part of the increased fibroblasts population observed in SSc skin could be derived from epithelial cells via EMT. Studies on organs such as lungs have suggested importance of EMT in SSc, however the scientific community is divided as to the existence of this process in organs other than lungs. Revealed in this thesis elevated levels of S100A9 and CTGF in SSc epidermis, alongside with the widely accepted increased TGFβ signaling and inflammatory microenvironment in the SSc skin could lead to the initiation of the EMT process (Sonnylal et al., 2013, Cormier et al., 2014). Indeed some of the changes presented in this thesis are consistent with a partial EMT like process and supplement previous work carried out on EMT in SSc skin (Gillespie et al., 2011, Nakamura and Tokura, 2011), as the current evidence and understanding of EMT in SSc skin is limited;

partly because of the need to lineage trace cells to show definitely that cells of epithelial origin are migrating into the dermis and taking on mesenchymal properties, which would depend on use of animal model systems.

Consistent with the phosphorylation array results this thesis reports increased phosphorylation of Smad2/3 in the epidermis and the papillary dermis, confirming cross talk between the activated epidermis and dermis. However, such enhanced TGF-β signalling did not lead to changes in expression of E-cadherin, type IV collagen of the basal membrane, FSP-1 or vimentin. Nevertheless, α-SMA was detected in the epidermis of some of the SSc patients, which along with increase in EMT-inducing transcription factor *SNAI1* mRNA may suggest a partially evolved EMT process in the SSc skin.

My work also shows that the expression pattern of pro-collagen type I in SSc skin changes with the disease duration. Early stage of SSc is characterized by extracellular expression, especially at the epidermal dermal junction whereas in the late stage the number of pro-collagen type I positive cells increase with a simultaneous reduction in the extracellular expression in the papillary dermis. This work contradicts previous report of pro-collagen in SSc skin showing increased staining within hypodermis in late SSc (Fleischmajer et al., 1980). Though the antibodies used in that study had multiple epitopes so more ECM staining was detected and the patients' subset (lcSSc or dcSSc) is not specified. Therefore it is hard to compare with the results generated using a more specific antibody. The fact that the changes are located in the papillary dermis suggests that there are issues with epidermal-dermal cross talk. To better understand the collagen metabolism in SSc skin and epidermal involvement in the process, the levels of the pro-peptidases responsible for the collagen processing should also be investigated. It would also be important to distinguish between the levels of free pro-collagen peptides and the unprocessed molecules.

Although active Smad mediated TGF-β signaling, enhanced CTGF expression, along with enhanced levels of Snail1 mRNA in SSc epidermis suggest EMT changes, other

results did not confirm EMT in the SSc skin. Therefore in contrary to earlier published work, data presented in this thesis does not support idea of complete EMT in the SSc epidermis and rather points to activation and altered terminal differentiation program, which partially resembles an EMT process. Such partial EMT has been described to occur during wound re-epithelialisation thus further strengthening the evidence of similarities between SSc epidermis and wound healing. Though I have successfully established the EMT model using HaCaT cells treated with TGFβ1 which showed changes consistent with those previously reported (Rasanen and Vaheri, 2010, Lamouille and Derynck, 2007, Wang et al., 2012, Herfs et al., 2008, Zavadil and Bottinger, 2005), insufficient number of primary keratinocytes prevented me from following the *in vitro* study on EMT using SSc and control keratinocytes. If I were not bound by the time constraints of this thesis, I would generate immortal SSc keratinocyte lines in order to study in more detail if SSc keratinocytes undergo EMT more easily than control cells, and test whether there is any evidence of more spontaneous events of EMT in cell culture between the disease and control keratinocytes.

# Chapter Seven

# References

1980. Preliminary criteria for the classification of systemic sclerosis (scleroderma). Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. *Arthritis Rheum,* 23**,** 581-90.

ABRAHAM, D. J., ECKES, B., RAJKUMAR, V. & KRIEG, T. 2007. New developments in fibroblast and myofibroblast biology: implications for fibrosis and scleroderma. *Curr Rheumatol Rep,* 9**,** 136-43.

ABRAHAM, D. J., SHIWEN, X., BLACK, C. M., SA, S., XU, Y. & LEASK, A. 2000. Tumor necrosis factor alpha suppresses the induction of connective tissue growth factor by transforming growth factor-beta in normal and scleroderma fibroblasts. *J Biol Chem,* 275**,** 15220-5.

ABRAHAM, D. J. & VARGA, J. 2005. Scleroderma: from cell and molecular mechanisms to disease models. *Trends Immunol,* 26**,** 587-95.

ADEN, N., NUTTALL, A., SHIWEN, X., DE WINTER, P., LEASK, A., BLACK, C. M., DENTON, C. P., ABRAHAM, D. J. & STRATTON, R. J. 2010. Epithelial cells promote fibroblast activation via IL-1alpha in systemic sclerosis. *J Invest Dermatol,* 130**,** 2191-200.

ADEN, N., SHIWEN, X., ADEN, D., BLACK, C., NUTTALL, A., DENTON, C. P., LEASK, A., ABRAHAM, D. & STRATTON, R. 2008. Proteomic analysis of scleroderma lesional skin reveals activated wound healing phenotype of epidermal cell layer. *Rheumatology.(Oxford),* 47**,** 1754-1760.

ALBANESI, C., SCARPONI, C., GIUSTIZIERI, M. L. & GIROLOMONI, G. 2005. Keratinocytes in inflammatory skin diseases. *Curr Drug Targets Inflamm Allergy,* 4**,** 329-34.

ALFORD, K. A., GLENNIE, S., TURRELL, B. R., RAWLINSON, L., SAKLATVALA, J. & DEAN, J. L. 2007. Heat shock protein 27 functions in inflammatory gene expression and transforming growth factor-beta-activated kinase-1 (TAK1)-mediated signaling. *J Biol Chem,* 282**,** 6232-41.

ALLANORE, Y., SAAD, M., DIEUDE, P., AVOUAC, J., DISTLER, J. H., AMOUYEL, P., MATUCCI-CERINIC, M., RIEMEKASTEN, G., AIRO, P., MELCHERS, I., HACHULLA, E., CUSI, D., WICHMANN, H. E., WIPFF, J., LAMBERT, J. C., HUNZELMANN, N., TIEV, K., CARAMASCHI, P., DIOT, E., KOWAL-BIELECKA, O., VALENTINI, G., MOUTHON, L., CZIRJAK, L., DAMJANOV, N., SALVI, E., CONTI, C., MULLER, M., MULLER-LADNER, U., RICCIERI, V., RUIZ, B., CRACOWSKI, J.

L., LETENNEUR, L., DUPUY, A. M., MEYER, O., KAHAN, A., MUNNICH, A., BOILEAU, C. & MARTINEZ, M. 2011. Genome-wide scan identifies TNIP1, PSORS1C1, and RHOB as novel risk loci for systemic sclerosis. *PLoS Genet,* 7**,** e1002091.

ANDREASSON, K., SCHEJA, A., SAXNE, T., OHLSSON, B. & HESSELSTRAND, R. 2011. Faecal calprotectin: a biomarker of gastrointestinal disease in systemic sclerosis. *J.Intern.Med.,* 270**,** 50-57.

ANNES, J. P., MUNGER, J. S. & RIFKIN, D. B. 2003. Making sense of latent TGFbeta activation. *J Cell Sci,* 116**,** 217-24.

ANSEL, J. C., TIESMAN, J. P., OLERUD, J. E., KRUEGER, J. G., KRANE, J. F., TARA, D. C., SHIPLEY, G. D., GILBERTSON, D., USUI, M. L. & HART, C. E. 1993. Human keratinocytes are a major source of cutaneous platelet-derived growth factor. *J Clin Invest,* 92**,** 671-8.

ARDERN-JONES, M. R., BLACK, A. P., BATEMAN, E. A. & OGG, G. S. 2007. Bacterial superantigen facilitates epithelial presentation of allergen to T helper 2 cells. *Proc Natl Acad Sci U S A,* 104**,** 5557-62.

AREND, W. P., PALMER, G. & GABAY, C. 2008. IL-1, IL-18, and IL-33 families of cytokines. *Immunol Rev,* 223**,** 20-38.

ARNOUX, V., NASSOUR, M., L'HELGOUALC'H, A., HIPSKIND, R. A. & SAVAGNER, P. 2008. Erk5 controls Slug expression and keratinocyte activation during wound healing. *Mol Biol Cell,* 19**,** 4738-49.

ARNSON, Y., AMITAL, H., GUIDUCCI, S., MATUCCI-CERINIC, M., VALENTINI, G., BARZILAI, O., MAYA, R. & SHOENFELD, Y. 2009. The role of infections in the immunopathogensis of systemic sclerosis--evidence from serological studies. *Ann N Y Acad Sci,* 1173**,** 627-32.

ARRIGO, A. P. 2000. sHsp as novel regulators of programmed cell death and tumorigenicity. *Pathol Biol (Paris),* 48**,** 280-8.

ARWERT, E. N., HOSTE, E. & WATT, F. M. 2012. Epithelial stem cells, wound healing and cancer. *Nat Rev Cancer,* 12**,** 170-80.

ASSASSI, S., WU, M., TAN, F. K., CHANG, J., GRAHAM, T. A., FURST, D. E., KHANNA, D., CHARLES, J., FERGUSON, E. C., FEGHALI-BOSTWICK, C. & MAYES, M. D. 2013.

Skin gene expression correlates of severity of interstitial lung disease in systemic sclerosis. *Arthritis Rheum,* 65**,** 2917-27.

AVERILL, M. M., BARNHART, S., BECKER, L., LI, X., HEINECKE, J. W., LEBOEUF, R. C., HAMERMAN, J. A., SORG, C., KERKHOFF, C. & BORNFELDT, K. E. 2011. S100A9 differentially modifies phenotypic states of neutrophils, macrophages, and dendritic cells: implications for atherosclerosis and adipose tissue inflammation. *Circulation,* 123**,** 1216-26.

BAARSMA, H. A., MENZEN, M. H., HALAYKO, A. J., MEURS, H., KERSTJENS, H. A. & GOSENS, R. 2011. beta-Catenin signaling is required for TGF-beta1-induced extracellular matrix production by airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol,* 301**,** L956-65.

BAKER, B. S., OVIGNE, J. M., POWLES, A. V., CORCORAN, S. & FRY, L. 2003. Normal keratinocytes express Toll-like receptors (TLRs) 1, 2 and 5: modulation of TLR expression in chronic plaque psoriasis. *Br.J.Dermatol.,* 148**,** 670-679.

BAL, V., MCINDOE, A., DENTON, G., HUDSON, D., LOMBARDI, G., LAMB, J. & LECHLER, R. 1990. Antigen presentation by keratinocytes induces tolerance in human T cells. *Eur J Immunol,* 20**,** 1893-7.

BANDO, M., HIROSHIMA, Y., KATAOKA, M., HERZBERG, M. C., ROSS, K. F., SHINOHARA, Y., YAMAMOTO, T., NAGATA, T. & KIDO, J. 2010. Modulation of calprotectin in human keratinocytes by keratinocyte growth factor and interleukin-1alpha. *Immunol Cell Biol,* 88**,** 328-33.

BANDO, M., HIROSHIMA, Y., KATAOKA, M., SHINOHARA, Y., HERZBERG, M. C., ROSS, K. F., NAGATA, T. & KIDO, J. 2007. Interleukin-1alpha regulates antimicrobial peptide expression in human keratinocytes. *Immunol.Cell Biol.,* 85**,** 532-537.

BANNO, T., ADACHI, M., MUKKAMALA, L. & BLUMENBERG, M. 2003. Unique keratinocyte-specific effects of interferon-gamma that protect skin from viruses, identified using transcriptional profiling. *Antivir Ther,* 8**,** 541-54.

BARGAGLI, E., OLIVIERI, C., CINTORINO, M., REFINI, R. M., BIANCHI, N., PRASSE, A. & ROTTOLI, P. 2011. Calgranulin B (S100A9/MRP14): a key molecule in idiopathic pulmonary fibrosis? *Inflammation,* 34**,** 85-91.

BARKER, J. N., MITRA, R. S., GRIFFITHS, C. E., DIXIT, V. M. & NICKOLOFF, B. J. 1991. Keratinocytes as initiators of inflammation. *Lancet,* 337**,** 211-4.

BARNES, J. & MAYES, M. D. 2012. Epidemiology of systemic sclerosis: incidence, prevalence, survival, risk factors, malignancy, and environmental triggers. *Curr Opin Rheumatol,* 24**,** 165-70.

BEIRNE, P., PANTELIDIS, P., CHARLES, P., WELLS, A. U., ABRAHAM, D. J., DENTON, C. P., WELSH, K. I., SHAH, P. L., DU BOIS, R. M. & KELLEHER, P. 2009. Multiplex immune serum biomarker profiling in sarcoidosis and systemic sclerosis. *Eur Respir J,* 34**,** 1376-82.

BELLEMARE, J., ROBERGE, C. J., BERGERON, D., LOPEZ-VALLE, C. A., ROY, M. & MOULIN, V. J. 2005. Epidermis promotes dermal fibrosis: role in the pathogenesis of hypertrophic scars. *J.Pathol.,* 206**,** 1-8.

BENNDORF, R., HAYESS, K., RYAZANTSEV, S., WIESKE, M., BEHLKE, J. & LUTSCH, G. 1994. Phosphorylation and supramolecular organization of murine small heat shock protein HSP25 abolish its actin polymerization-inhibiting activity. *J Biol Chem,* 269**,** 20780-4.

BENOIT, S., TOKSOY, A., AHLMANN, M., SCHMIDT, M., SUNDERKOTTER, C., FOELL, D., PASPARAKIS, M., ROTH, J. & GOEBELER, M. 2006. Elevated serum levels of calcium-binding S100 proteins A8 and A9 reflect disease activity and abnormal differentiation of keratinocytes in psoriasis. *Br J Dermatol,* 155**,** 62-6.

BERNTZEN, H. B., MUNTHE, E. & FAGERHOL, M. K. 1989. A longitudinal study of the leukocyte protein L1 as an indicator of disease activity in patients with rheumatoid arthritis. *J Rheumatol,* 16**,** 1416-20.

BHATTACHARYYA, S., KELLEY, K., MELICHIAN, D. S., TAMAKI, Z., FANG, F., SU, Y., FENG, G., POPE, R. M., BUDINGER, G. R. S., MUTLU, G. M., LAFYATIS, R., RADSTAKE, T., FEGHALI-BOSTWICK, C. & VARGA, J. 2013. Toll-Like Receptor 4 Signaling Augments Transforming Growth Factor-β Responses: A Novel Mechanism for Maintaining and Amplifying Fibrosis in Scleroderma. *Am J Pathol,* 182**,** 192-205.

BHOWMICK, N. A., CHYTIL, A., PLIETH, D., GORSKA, A. E., DUMONT, N., SHAPPELL, S., WASHINGTON, M. K., NEILSON, E. G. & MOSES, H. L. 2004. TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. *Science,* 303**,** 848-51.

BIEBER, T., DANNENBERG, B., RING, J. & BRAUN-FALCO, O. 1989. Keratinocytes in lesional skin of atopic eczema bear HLA-DR, CD1a and IgE molecules. *Clin Exp Dermatol,* 14**,** 35-9.

BIENKOWSKI, R. S., BAUM, B. J. & CRYSTAL, R. G. 1978. Fibroblasts degrade newly synthesised collagen within the cell before secretion. *Nature,* 276**,** 413-6.

BJORK, P., BJORK, A., VOGL, T., STENSTROM, M., LIBERG, D., OLSSON, A., ROTH, J., IVARS, F. & LEANDERSON, T. 2009. Identification of human S100A9 as a novel target for treatment of autoimmune disease via binding to quinoline-3-carboxamides. *PLoS Biol,* 7**,** e97.

BLACK, A. P., ARDERN-JONES, M. R., KASPROWICZ, V., BOWNESS, P., JONES, L., BAILEY, A. S. & OGG, G. S. 2007. Human keratinocyte induction of rapid effector function in antigen-specific memory CD4+ and CD8+ T cells. *Eur J Immunol,* 37**,** 1485-93.

BOGATKEVICH, G. S., LUDWICKA-BRADLEY, A., HIGHLAND, K. B., HANT, F., NIETERT, P. J., SINGLETON, C. B. & SILVER, R. M. 2007. Down-regulation of collagen and connective tissue growth factor expression with hepatocyte growth factor in lung fibroblasts from white scleroderma patients via two signaling pathways. *Arthritis & Rheumatism,* 56**,** 3468-3477.

BOGUMIL, T., RIECKMANN, P., KUBUSCHOK, B., FELGENHAUER, K. & BRUCK, W. 1998. Serum levels of macrophage-derived protein MRP-8/14 are elevated in active multiple sclerosis. *Neurosci Lett,* 247**,** 195-7.

BONI, R., BURG, G., DOGUOGLU, A., ILG, E. C., SCHAFER, B. W., MULLER, B. & HEIZMANN, C. W. 1997. Immunohistochemical localization of the Ca2+ binding S100 proteins in normal human skin and melanocytic lesions. *Br J Dermatol,* 137**,** 39-43.

BONIFACE, K., BERNARD, F. X., GARCIA, M., GURNEY, A. L., LECRON, J. C. & MOREL, F. 2005. IL-22 inhibits epidermal differentiation and induces proinflammatory gene expression and migration of human keratinocytes. *J Immunol,* 174**,** 3695-702.

BONISH, B., JULLIEN, D., DUTRONC, Y., HUANG, B. B., MODLIN, R., SPADA, F. M., PORCELLI, S. A. & NICKOLOFF, B. J. 2000. Overexpression of CD1d by keratinocytes in psoriasis and CD1d-dependent IFN-gamma production by NK-T cells. *J Immunol,* 165**,** 4076-85.

BONNER, J. C. 2004. Regulation of PDGF and its receptors in fibrotic diseases. *Cytokine Growth Factor Rev,* 15**,** 255-73.

BONNIAUD, P., KOLB, M., GALT, T., ROBERTSON, J., ROBBINS, C., STAMPFLI, M., LAVERY, C., MARGETTS, P. J., ROBERTS, A. B. & GAULDIE, J. 2004. Smad3 null mice develop airspace enlargement and are resistant to TGF-beta-mediated pulmonary fibrosis. *J Immunol,* 173**,** 2099-108.

BOUKAMP, P., PETRUSSEVSKA, R. T., BREITKREUTZ, D., HORNUNG, J., MARKHAM, A. & FUSENIG, N. E. 1988. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J Cell Biol,* 106**,** 761-71.

BOUKAMP, P., RUPNIAK, H. T. & FUSENIG, N. E. 1985. Environmental modulation of the expression of differentiation and malignancy in six human squamous cell carcinoma cell lines. *Cancer Res,* 45**,** 5582-92.

BOYCE & HAM 1985. Cultivation frozen storage, and clonal growth of normal human keratinocytes in serum free media. *J Tissue Cult Meth,* 9**,** 83-93.

BOYCE, S. T. & HAM, R. G. 1983. Calcium-Regulated Differentiation of Normal Human Epidermal Keratinocytes in Chemically Defined Clonal Culture and Serum-Free Serial Culture. *J Investig Dermatol,* 81**,** 33s-40s.

BREZNICEANU, M. L., VOLP, K., BOSSER, S., SOLBACH, C., LICHTER, P., JOOS, S. & ZORNIG, M. 2003. HMGB1 inhibits cell death in yeast and mammalian cells and is abundantly expressed in human breast carcinoma. *FASEB J,* 17**,** 1295-7.

BROOME, A. M., RYAN, D. & ECKERT, R. L. 2003. S100 protein subcellular localization during epidermal differentiation and psoriasis. *J Histochem Cytochem,* 51**,** 675-85.

BURWINKEL, F., ROTH, J., GOEBELER, M., BITTER, U., WROCKLAGE, V., VOLLMER, E., ROESSNER, A., SORG, C. & BOCKER, W. 1994. Ultrastructural localization of the S-100-like proteins MRP8 and MRP14 in monocytes is calcium-dependent. *Histochemistry,* 101**,** 113-20.

CANDI, E., SCHMIDT, R. & MELINO, G. 2005. The cornified envelope: a model of cell death in the skin. *Nat Rev Mol Cell Biol,* 6**,** 328-340.

CARULLI, M. T., HANDLER, C., COGHLAN, J. G., BLACK, C. M. & DENTON, C. P. 2008. Can CCL2 serum levels be used in risk stratification or to monitor treatment response in systemic sclerosis? *Ann Rheum Dis,* 67**,** 105-9.

CELLI, A., MACKENZIE, D. S., CRUMRINE, D. S., TU, C. L., HUPE, M., BIKLE, D. D., ELIAS, P. M. & MAURO, T. M. 2011. Endoplasmic reticulum Ca2+ depletion activates XBP1 and controls terminal differentiation in keratinocytes and epidermis. *Br.J.Dermatol.,* 164**,** 16-25.

CESARO, A., ANCERIZ, N., PLANTE, A., PAGE, N., TARDIF, M. R. & TESSIER, P. A. 2012. An Inflammation Loop Orchestrated by S100A9 and Calprotectin Is Critical for Development of Arthritis. *PLoS One,* 7**,** e45478.

CHAO, Y., WU, Q., ACQUAFONDATA, M., DHIR, R. & WELLS, A. 2012. Partial mesenchymal to epithelial reverting transition in breast and prostate cancer metastases. *Cancer Microenviron,* 5**,** 19-28.

CHEN, C. C., CHEN, N. & LAU, L. F. 2001. The angiogenic factors Cyr61 and connective tissue growth factor induce adhesive signaling in primary human skin fibroblasts. *J Biol Chem,* 276**,** 10443-52.

CHEN, Z. Y., MARICQ, H. R., PILIA, P. A., DOBSON, R. L., SILVER, R. M. & AINSWORTH, S. K. 1985. Fibronectin distribution in nailfold biopsies of scleroderma (systemic sclerosis) patients. *Acta Derm.Venereol.,* 65**,** 185-189.

CHENG, C., GUO, G. F., MARTINEZ, J. A., SINGH, V. & ZOCHODNE, D. W. 2010. Dynamic plasticity of axons within a cutaneous milieu. *J Neurosci,* 30**,** 14735-44.

CHENG, P., CORZO, C. A., LUETTEKE, N., YU, B., NAGARAJ, S., BUI, M. M., ORTIZ, M., NACKEN, W., SORG, C., VOGL, T., ROTH, J. & GABRILOVICH, D. I. 2008. Inhibition of dendritic cell differentiation and accumulation of myeloid-derived suppressor cells in cancer is regulated by S100A9 protein. *J Exp Med,* 205**,** 2235-49.

CHILOSI, M., POLETTI, V., ZAMO, A., LESTANI, M., MONTAGNA, L., PICCOLI, P., PEDRON, S., BERTASO, M., SCARPA, A., MURER, B., CANCELLIERI, A., MAESTRO, R., SEMENZATO, G. & DOGLIONI, C. 2003. Aberrant Wnt/beta-catenin pathway activation in idiopathic pulmonary fibrosis. *Am J Pathol,* 162**,** 1495-502.

CHIN, G. S., LIU, W., PELED, Z., LEE, T. Y., STEINBRECH, D. S., HSU, M. & LONGAKER, M. T. 2001. Differential expression of transforming growth factor-beta receptors I and II and activation of Smad 3 in keloid fibroblasts. *Plast Reconstr Surg,* 108**,** 423-9.

CHIQUET, M., GELMAN, L., LUTZ, R. & MAIER, S. 2009. From mechanotransduction to extracellular matrix gene expression in fibroblasts. *Biochim Biophys Acta,* 1793**,** 911-20.

CHRISTMANN, R. B., SAMPAIO-BARROS, P., STIFANO, G., BORGES, C. L., DE CARVALHO, C. R., KAIRALLA, R., PARRA, E. R., SPIRA, A., SIMMS, R., CAPELLOZZI, V. L. & LAFYATIS, R. 2014. Association of Interferon- and transforming growth factor beta-regulated genes and macrophage activation with systemic sclerosis-related progressive lung fibrosis. *Arthritis Rheumatol,* 66**,** 714-25.

CLEMENTS, P., LACHENBRUCH, P., SIEBOLD, J., WHITE, B., WEINER, S., MARTIN, R., WEINSTEIN, A., WEISMAN, M., MAYES, M., COLLIER, D. & ET AL. 1995. Inter and intraobserver variability of total skin thickness score (modified Rodnan TSS) in systemic sclerosis. *J Rheumatol,* 22**,** 1281-5.

CLEMENTS, P. J., HURWITZ, E. L., WONG, W. K., SEIBOLD, J. R., MAYES, M., WHITE, B., WIGLEY, F., WEISMAN, M., BARR, W., MORELAND, L., MEDSGER, T. A., JR., STEEN, V. D., MARTIN, R. W., COLLIER, D., WEINSTEIN, A., LALLY, E., VARGA, J., WEINER, S. R., ANDREWS, B., ABELES, M. & FURST, D. E. 2000. Skin thickness score as a predictor and correlate of outcome in systemic sclerosis: high-dose versus low-dose penicillamine trial. *Arthritis Rheum,* 43**,** 2445-54.

CLEMENTS, P. J., LACHENBRUCH, P. A., NG, S. C., SIMMONS, M., STERZ, M. & FURST, D. E. 1990. Skin score. A semiquantitative measure of cutaneous involvement that improves prediction of prognosis in systemic sclerosis. *Arthritis Rheum,* 33**,** 1256-63.

CLEMENTS, P. J., LACHENBRUCH, P. A., SEIBOLD, J. R., ZEE, B., STEEN, V. D., BRENNAN, P., SILMAN, A. J., ALLEGAR, N., VARGA, J., MASSA, M. & ET AL. 1993. Skin thickness score in systemic sclerosis: an assessment of interobserver variability in 3 independent studies. *J Rheumatol,* 20**,** 1892-6.

CODULLO, V., BALDWIN, H. M., SINGH, M. D., FRASER, A. R., WILSON, C., GILMOUR, A., HUEBER, A. J., BONINO, C., MCINNES, I. B., MONTECUCCO, C. & GRAHAM, G. J. 2011. An investigation of the inflammatory cytokine and chemokine network in systemic sclerosis. *Ann Rheum Dis,* 70**,** 1115-21.

CONNELLY, J. T., GAUTROT, J. E., TRAPPMANN, B., TAN, D. W., DONATI, G., HUCK, W. T. & WATT, F. M. 2010. Actin and serum response factor transduce physical cues from the microenvironment to regulate epidermal stem cell fate decisions. *Nat.Cell Biol.,* 12**,** 711-718.

COOPER, S. M., KEYSER, A. J., BEAULIEU, A. D., RUOSLAHTI, E., NIMNI, M. E. & QUISMORIO, F. P., JR. 1979. Increase in fibronectin in the deep dermis of involved skin in progressive systemic sclerosis. *Arthritis Rheum,* 22**,** 983-7.

CORMIER, K., HARQUAIL, J., OUELLETTE, R. J., TESSIER, P. A., GUERRETTE, R. & ROBICHAUD, G. A. 2014. Intracellular Expression of Inflammatory Proteins S100A8 and S100A9 Leads to Epithelial-mesenchymal Transition and Attenuated Aggressivity of Breast Cancer Cells. *Anticancer Agents Med Chem,* 14**,** 35-45.

COTTRELL, T. R., WISE, R. A., WIGLEY, F. M. & BOIN, F. 2014. The degree of skin involvement identifies distinct lung disease outcomes and survival in systemic sclerosis. *Ann Rheum Dis,* 73**,** 1060-6.

COWIE, R. L. 1987. Silica-dust-exposed mine workers with scleroderma (systemic sclerosis). *Chest,* 92**,** 260-2.

CREAN, J. K., FINLAY, D., MURPHY, M., MOSS, C., GODSON, C., MARTIN, F. & BRADY, H. R. 2002. The role of p42/44 MAPK and protein kinase B in connective tissue growth factor induced extracellular matrix protein production, cell migration, and actin cytoskeletal rearrangement in human mesangial cells. *J Biol Chem,* 277**,** 44187-94.

CROSS, S. S., HAMDY, F. C., DELOULME, J. C. & REHMAN, I. 2005. Expression of S100 proteins in normal human tissues and common cancers using tissue microarrays: S100A6, S100A8, S100A9 and S100A11 are all overexpressed in common cancers. *Histopathology,* 46**,** 256-69.

CROWE, J., AUBAREDA, A., MCNAMEE, K., PRZYBYCIEN, P. M., LU, X., WILLIAMS, R. O., BOU-GHARIOS, G., SAKLATVALA, J. & DEAN, J. L. 2013. Heat shock protein B1-deficient mice display impaired wound healing. *PLoS One,* 8**,** e77383.

DALRYMPLE, S. L., BECKER, R. E., ZHOU, H., DEWEESE, T. L. & ISAACS, J. T. 2012. Tasquinimod prevents the angiogenic rebound induced by fractionated radiation resulting in an enhanced therapeutic response of prostate cancer xenografts. *Prostate,* 72**,** 638-48.

DAVIES, C. A., HERRICK, A. L., CORDINGLEY, L., FREEMONT, A. J. & JEZIORSKA, M. 2009. Expression of advanced glycation end products and their receptor in skin from patients with systemic sclerosis with and without calcinosis. *Rheumatology (Oxford),* 48**,** 876-82.

DAVIES, C. A., JEZIORSKA, M., FREEMONT, A. J. & HERRICK, A. L. 2006. The differential expression of VEGF, VEGFR-2, and GLUT-1 proteins in disease subtypes of systemic sclerosis. *Hum.Pathol.,* 37**,** 190-197.

DAZARD, J. E., GAL, H., AMARIGLIO, N., RECHAVI, G., DOMANY, E. & GIVOL, D. 2003. Genome-wide comparison of human keratinocyte and squamous cell carcinoma responses to UVB irradiation: implications for skin and epithelial cancer. *Oncogene,* 22**,** 2993-3006.

DE WAAL, R. M., SEMEIJN, J. T., CORNELISSEN, M. H. & RAMAEKERS, F. C. 1984. Epidermal Langerhans cells contain intermediate-sized filaments of the vimentin type: an immunocytologic study. *J Invest Dermatol,* 82**,** 602-4.

DE WINTER, P., LEONI, P. & ABRAHAM, D. 2008. Connective tissue growth factor: structure-function relationships of a mosaic, multifunctional protein. *Growth Factors,* 26**,** 80-91.

DEMARCO, P. J., WEISMAN, M. H., SEIBOLD, J. R., FURST, D. E., WONG, W. K., HURWITZ, E. L., MAYES, M., WHITE, B., WIGLEY, F., BARR, W., MORELAND, L., MEDSGER, T. A., JR., STEEN, V., MARTIN, R. W., COLLIER, D., WEINSTEIN, A., LALLY, E., VARGA, J., WEINER, S. R., ANDREWS, B., ABELES, M. & CLEMENTS, P. J. 2002. Predictors and outcomes of scleroderma renal crisis: the high-dose versus low-dose D-penicillamine in early diffuse systemic sclerosis trial. *Arthritis Rheum,* 46**,** 2983-9.

DENTON, C. P. & ABRAHAM, D. J. 2001. Transforming growth factor-beta and connective tissue growth factor: key cytokines in scleroderma pathogenesis. *Curr Opin Rheumatol,* 13**,** 505-11.

DENTON, C. P. & BLACK, C. M. 2004. Scleroderma--clinical and pathological advances. *Best Pract Res Clin Rheumatol,* 18**,** 271-90.

DENTON, C. P., KHAN, K., HOYLES, R. K., SHIWEN, X., LEONI, P., CHEN, Y., EASTWOOD, M. & ABRAHAM, D. J. 2009. Inducible lineage-specific deletion of TbetaRII in fibroblasts defines a pivotal regulatory role during adult skin wound healing. *J Invest Dermatol,* 129**,** 194-204.

DENTON, C. P. & ONG, V. H. 2013. Targeted therapies for systemic sclerosis. *Nat Rev Rheumatol,* 9**,** 451-64.

DERRETT-SMITH, E. C. 2013. *Systemic sclerosis vasculopathy : exploration in transgenic mice*.

DESMOULIERE, A., CHAPONNIER, C. & GABBIANI, G. 2005. Tissue repair, contraction, and the myofibroblast. *Wound Repair Regen,* 13**,** 7-12.

DESMOULIERE, A., DARBY, I. A. & GABBIANI, G. 2003. Normal and pathologic soft tissue remodeling: role of the myofibroblast, with special emphasis on liver and kidney fibrosis. *Lab Invest,* 83**,** 1689-707.

DIAZ-GALLO, L. M., GOURH, P., BROEN, J., SIMEON, C., FONOLLOSA, V., ORTEGO-CENTENO, N., AGARWAL, S., VONK, M. C., COENEN, M., RIEMEKASTEN, G., HUNZELMANN, N., HESSELSTRAND, R., TAN, F. K., REVEILLE, J. D., ASSASSI, S., GARCIA-HERNANDEZ, F. J., CARREIRA, P., CAMPS, M. T., FERNANDEZ-NEBRO, A., DE LA PENA, P. G., NEARNEY, T., HILDA, D., GONZALEZ-GAY, M. A., AIRO, P., BERETTA, L., SCORZA, R., HERRICK, A., WORTHINGTON, J., PROS, A., GOMEZ-GRACIA, I., TRAPIELLA, L., ESPINOSA, G., CASTELLVI, I., WITTE, T., DE KEYSER, F., VANTHUYNE, M., MAYES, M. D., RADSTAKE, T. R., ARNETT, F. C., MARTIN, J. & RUEDA, B. 2011. Analysis of the influence of PTPN22 gene polymorphisms in systemic sclerosis. *Ann Rheum Dis,* 70**,** 454-62.

DIEU-NOSJEAN, M. C., MASSACRIER, C., HOMEY, B., VANBERVLIET, B., PIN, J. J., VICARI, A., LEBECQUE, S., DEZUTTER-DAMBUYANT, C., SCHMITT, D., ZLOTNIK, A. & CAUX, C. 2000. Macrophage inflammatory protein 3alpha is expressed at inflamed epithelial surfaces and is the most potent chemokine known in attracting Langerhans cell precursors. *J Exp Med,* 192**,** 705-18.

DIEUDE, P., BOILEAU, C., GUEDJ, M., AVOUAC, J., RUIZ, B., HACHULLA, E., DIOT, E., CRACOWSKI, J. L., TIEV, K., SIBILIA, J., MOUTHON, L., FRANCES, C., AMOURA, Z., CARPENTIER, P., COSNES, A., MEYER, O., KAHAN, A., CHIOCCHIA, G. & ALLANORE, Y. 2011. Independent replication establishes the CD247 gene as a genetic systemic sclerosis susceptibility factor. *Ann Rheum Dis,* 70**,** 1695-6.

DIEUDE, P., DAWIDOWICZ, K., GUEDJ, M., LEGRAIN, Y., WIPFF, J., HACHULLA, E., DIOT, E., SIBILIA, J., MOUTHON, L., CABANE, J., AMOURA, Z., CRAKOWSKI, J. L., CARPENTIER, P., AVOUAC, J., MEYER, O., KAHAN, A., BOILEAU, C. & ALLANORE, Y. 2010. Phenotype-haplotype correlation of IRF5 in systemic sclerosis: role of 2 haplotypes in disease severity. *J Rheumatol,* 37**,** 987-92.

DIEUDE, P., GUEDJ, M., WIPFF, J., AVOUAC, J., HACHULLA, E., DIOT, E., GRANEL, B., SIBILIA, J., CABANE, J., MEYER, O., MOUTHON, L., KAHAN, A., BOILEAU, C. & ALLANORE, Y. 2008. The PTPN22 620W allele confers susceptibility to systemic sclerosis: findings of a large case-control study of European Caucasians and a meta-analysis. *Arthritis Rheum,* 58**,** 2183-8.

DIEUDE, P., GUEDJ, M., WIPFF, J., RUIZ, B., HACHULLA, E., DIOT, E., GRANEL, B., SIBILIA, J., TIEV, K., MOUTHON, L., CRACOWSKI, J. L., CARPENTIER, P. H., AMOURA, Z., FAJARDY, I., AVOUAC, J., MEYER, O., KAHAN, A., BOILEAU, C. & ALLANORE, Y. 2009a. STAT4 is a genetic risk factor for systemic sclerosis having additive effects with IRF5 on disease susceptibility and related pulmonary fibrosis. *Arthritis Rheum,* 60**,** 2472-9.

DIEUDE, P., WIPFF, J., GUEDJ, M., RUIZ, B., MELCHERS, I., HACHULLA, E., RIEMEKASTEN, G., DIOT, E., HUNZELMANN, N., SIBILIA, J., TIEV, K., MOUTHON, L., CRACOWSKI, J. L., CARPENTIER, P. H., DISTLER, J., AMOURA, Z., TARNER, I., AVOUAC, J., MEYER, O., KAHAN, A., BOILEAU, C. & ALLANORE, Y. 2009b. BANK1 is a genetic risk factor for diffuse cutaneous systemic sclerosis and has additive effects with IRF5 and STAT4. *Arthritis Rheum,* 60**,** 3447-54.

DISTLER, J. H., JUNGEL, A., KOWAL-BIELECKA, O., MICHEL, B. A., GAY, R. E., SPROTT, H., MATUCCI-CERINIC, M., CHILLA, M., REICH, K., KALDEN, J. R., MULLER-LADNER, U., LORENZ, H. M., GAY, S. & DISTLER, O. 2005. Expression of interleukin-21 receptor in epidermis from patients with systemic sclerosis. *Arthritis Rheum.,* 52**,** 856-864.

DISTLER, O., PAP, T., KOWAL-BIELECKA, O., MEYRINGER, R., GUIDUCCI, S., LANDTHALER, M., SCHOLMERICH, J., MICHEL, B. A., GAY, R. E., MATUCCI-CERINIC, M., GAY, S. & MULLER-LADNER, U. 2001. Overexpression of monocyte chemoattractant protein 1 in systemic sclerosis: role of platelet-derived growth factor and effects on monocyte chemotaxis and collagen synthesis. *Arthritis Rheum.,* 44**,** 2665-2678.

DONG, C., ZHU, S., WANG, T., YOON, W., LI, Z., ALVAREZ, R. J., TEN DIJKE, P., WHITE, B., WIGLEY, F. M. & GOLDSCHMIDT-CLERMONT, P. J. 2002. Deficient Smad7 expression: a putative molecular defect in scleroderma. *Proc Natl Acad Sci U S A,* 99**,** 3908-13.

DZIADZIO, M., SMITH, R. E., ABRAHAM, D. J., BLACK, C. M. & DENTON, C. P. 2005. Circulating levels of active transforming growth factor beta1 are reduced in diffuse cutaneous systemic sclerosis and correlate inversely with the modified Rodnan skin score. *Rheumatology (Oxford),* 44**,** 1518-24.

ECKERT, R. L., EFIMOVA, T., DASHTI, S. R., BALASUBRAMANIAN, S., DEUCHER, A., CRISH, J. F., STURNIOLO, M. & BONE, F. 2002. Keratinocyte survival, differentiation, and death: many roads lead to mitogen-activated protein kinase. *J.Investig.Dermatol.Symp.Proc.,* 7**,** 36-40.

ECKES, B., NISCHT, R. & KRIEG, T. 2010. Cell-matrix interactions in dermal repair and scarring. *Fibrogenesis Tissue Repair,* 3**,** 4.

EL GHALBZOURI, A. & PONEC, M. 2004. Diffusible factors released by fibroblasts support epidermal morphogenesis and deposition of basement membrane components. *Wound Repair Regen,* 12**,** 359-67.

FALANGA, V., GERHARDT, C. O., DASCH, J. R., TAKEHARA, K. & KSANDER, G. A. 1992. Skin distribution and differential expression of transforming growth factor beta 1 and beta 2. *J Dermatol Sci,* 3**,** 131-6.

FALER, B. J., MACSATA, R. A., PLUMMER, D., MISHRA, L. & SIDAWY, A. N. 2006. Transforming growth factor-beta and wound healing. *Perspect Vasc Surg Endovasc Ther,* 18**,** 55-62.

FARINA, A., CIRONE, M., YORK, M., LENNA, S., PADILLA, C., MCLAUGHLIN, S., FAGGIONI, A., LAFYATIS, R., TROJANOWSKA, M. & FARINA, G. A. 2014. Epstein-Barr virus infection induces aberrant TLR activation pathway and fibroblast-myofibroblast conversion in scleroderma. *J Invest Dermatol,* 134**,** 954-64.

FARINA, G., LEMAIRE, R., PANCARI, P., BAYLE, J., WIDOM, R. L. & LAFYATIS, R. 2009. Cartilage oligomeric matrix protein expression in systemic sclerosis reveals heterogeneity of dermal fibroblast responses to transforming growth factor beta. *Ann Rheum Dis,* 68**,** 435-41.

FLANDERS, K. C., SULLIVAN, C. D., FUJII, M., SOWERS, A., ANZANO, M. A., ARABSHAHI, A., MAJOR, C., DENG, C., RUSSO, A., MITCHELL, J. B. & ROBERTS, A. B. 2002. Mice lacking Smad3 are protected against cutaneous injury induced by ionizing radiation. *Am J Pathol,* 160**,** 1057-68.

FLAUMENHAFT, R. & RIFKIN, D. B. 1991. Extracellular matrix regulation of growth factor and protease activity. *Curr Opin Cell Biol,* 3**,** 817-23.

FLEISCHMAJER, R., DESSAU, W., TIMPL, R., KRIEG, T., LUDERSCHMIDT, C. & WIESTNER, M. 1980. Immunofluorescence analysis of collagen, fibronectin, and basement membrane protein in scleroderma skin. *J Invest Dermatol,* 75**,** 270-4.

FLIER, S. N., TANJORE, H., KOKKOTOU, E. G., SUGIMOTO, H., ZEISBERG, M. & KALLURI, R. 2010. Identification of epithelial to mesenchymal transition as a

novel source of fibroblasts in intestinal fibrosis. *J.Biol.Chem.,* 285**,** 20202-20212.

FOELL, D., WITTKOWSKI, H., VOGL, T. & ROTH, J. 2007. S100 proteins expressed in phagocytes: a novel group of damage-associated molecular pattern molecules. *J Leukoc Biol,* 81**,** 28-37.

FONSECA, C., LINDAHL, G. E., PONTICOS, M., SESTINI, P., RENZONI, E. A., HOLMES, A. M., SPAGNOLO, P., PANTELIDIS, P., LEONI, P., MCHUGH, N., STOCK, C. J., SHI-WEN, X., DENTON, C. P., BLACK, C. M., WELSH, K. I., DU BOIS, R. M. & ABRAHAM, D. J. 2007. A polymorphism in the CTGF promoter region associated with systemic sclerosis. *N Engl J Med,* 357**,** 1210-20.

FOUSER, L., SAGE, E. H., CLARK, J. & BORNSTEIN, P. 1991. Feedback regulation of collagen gene expression: a Trojan horse approach. *Proc Natl Acad Sci U S A,* 88**,** 10158-62.

FRANCESCHINI, A., SZKLARCZYK, D., FRANKILD, S., KUHN, M., SIMONOVIC, M., ROTH, A., LIN, J., MINGUEZ, P., BORK, P., VON MERING, C. & JENSEN, L. J. 2013. STRING v9.1: protein-protein interaction networks, with increased coverage and integration. *Nucleic Acids Res,* 41**,** D808-15.

FRAZIER, K., WILLIAMS, S., KOTHAPALLI, D., KLAPPER, H. & GROTENDORST, G. R. 1996. Stimulation of fibroblast cell growth, matrix production, and granulation tissue formation by connective tissue growth factor. *J Invest Dermatol,* 107**,** 404-11.

FROST, J., RAMSAY, M., MIA, R., MOOSA, L., MUSENGE, E. & TIKLY, M. 2012. Differential gene expression of MMP-1, TIMP-1 and HGF in clinically involved and uninvolved skin in South Africans with SSc. *Rheumatology (Oxford)*.

FUCHS, E. 1990. Epidermal differentiation. *Curr Opin Cell Biol,* 2**,** 1028-35.

FUNAYAMA, E., CHODON, T., OYAMA, A. & SUGIHARA, T. 2003. Keratinocytes promote proliferation and inhibit apoptosis of the underlying fibroblasts: an important role in the pathogenesis of keloid. *J Invest Dermatol,* 121**,** 1326-31.

FURST, D. E., CLEMENTS, P. J., STEEN, V. D., MEDSGER, T. A., JR., MASI, A. T., D'ANGELO, W. A., LACHENBRUCH, P. A., GRAU, R. G. & SEIBOLD, J. R. 1998. The modified Rodnan skin score is an accurate reflection of skin biopsy thickness in systemic sclerosis. *J Rheumatol,* 25**,** 84-8.

GABBIANI, G., RYAN, G. B. & MAJNE, G. 1971. Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. *Experientia,* 27**,** 549-50.

GAILIT, J. & CLARK, R. A. 1994. Wound repair in the context of extracellular matrix. *Curr Opin Cell Biol,* 6**,** 717-25.

GANDOUR-EDWARDS, R., MCCLAREN, M. & ISSEROFF, R. R. 1994. Immunolocalization of low-molecular-weight stress protein HSP 27 in normal skin and common cutaneous lesions. *Am J Dermatopathol,* 16**,** 504-9.

GASPARI, A. A., JENKINS, M. K. & KATZ, S. I. 1988. Class II MHC-bearing keratinocytes induce antigen-specific unresponsiveness in hapten-specific Th1 clones. *J Immunol,* 141**,** 2216-20.

GAY, S., JONES, R. E., JR., HUANG, G. Q. & GAY, R. E. 1989. Immunohistologic demonstration of platelet-derived growth factor (PDGF) and sis-oncogene expression in scleroderma. *J Invest Dermatol,* 92**,** 301-3.

GAZEL, A., BANNO, T., WALSH, R. & BLUMENBERG, M. 2006. Inhibition of JNK promotes differentiation of epidermal keratinocytes. *J.Biol.Chem.,* 281**,** 20530-20541.

GERRITSEN, M. E., TOMLINSON, J. E., ZLOT, C., ZIMAN, M. & HWANG, S. 2003. Using gene expression profiling to identify the molecular basis of the synergistic actions of hepatocyte growth factor and vascular endothelial growth factor in human endothelial cells. *Br J Pharmacol,* 140**,** 595-610.

GHAFFARI, A., KILANI, R. T. & GHAHARY, A. 2009. Keratinocyte-conditioned media regulate collagen expression in dermal fibroblasts. *J Invest Dermatol,* 129**,** 340-7.

GHAHARY, A., KARIMI-BUSHERI, F., MARCOUX, Y., LI, Y., TREDGET, E. E., TAGHI KILANI, R., LI, L., ZHENG, J., KARAMI, A., KELLER, B. O. & WEINFELD, M. 2004. Keratinocyte-releasable stratifin functions as a potent collagenase-stimulating factor in fibroblasts. *J Invest Dermatol,* 122**,** 1188-97.

GHARAEE-KERMANI, M., DENHOLM, E. M. & PHAN, S. H. 1996. Costimulation of fibroblast collagen and transforming growth factor beta1 gene expression by monocyte chemoattractant protein-1 via specific receptors. *J Biol Chem,* 271**,** 17779-84.

GILBOA, L., WELLS, R. G., LODISH, H. F. & HENIS, Y. I. 1998. Oligomeric structure of type I and type II transforming growth factor beta receptors: homodimers form in the ER and persist at the plasma membrane. *J Cell Biol,* 140**,** 767-77.

GILCHRIST, F. C., BUNN, C., FOLEY, P. J., LYMPANY, P. A., BLACK, C. M., WELSH, K. I. & DU BOIS, R. M. 2001. Class II HLA associations with autoantibodies in scleroderma: a highly significant role for HLA-DP. *Genes Immun,* 2**,** 76-81.

GILLESPIE, J., TINAZZI, I., COLATO, C., BENEDETTI, F., BIASI, D., CARAMASCHI, P., EMERY, P. & GALDO, F. D. 2011. Epithelial cells undergoing epithelial mesenchymal transition (EMT) in systemic sclerosis lack caveolin-1 and modulate WNT signaling in the dermis by secreting SFRP4. *Annals of the Rheumatic Diseases,* 70**,** A31-A32.

GILLIET, M. & LANDE, R. 2008. Antimicrobial peptides and self-DNA in autoimmune skin inflammation. *Curr Opin Immunol,* 20**,** 401-7.

GILLITZER, R. & GOEBELER, M. 2001. Chemokines in cutaneous wound healing. *J Leukoc Biol,* 69**,** 513-21.

GIUSTI, L., BAZZICHI, L., BALDINI, C., CIREGIA, F., MASCIA, G., GIANNACCINI, G., DEL, R. M., BOMBARDIERI, S. & LUCACCHINI, A. 2007. Specific proteins identified in whole saliva from patients with diffuse systemic sclerosis. *J.Rheumatol.,* 34**,** 2063-2069.

GOEBELER, M., ROTH, J., VAN DEN BOS, C., ADER, G. & SORG, C. 1995. Increase of calcium levels in epithelial cells induces translocation of calcium-binding proteins migration inhibitory factor-related protein 8 (MRP8) and MRP14 to keratin intermediate filaments. *Biochem J,* 309 ( Pt 2)**,** 419-24.

GRANEL, B., ARGIRO, L., HACHULLA, E., FAJARDY, I., WEILLER, P. J., DURAND, J. M., FRANCES, Y., DOMBEY, A. M., MARQUET, S., LESAVRE, N., DISDIER, P., BERNARD, F., HATRON, P. Y. & CHEVILLARD, C. 2010. Association between a CTGF gene polymorphism and systemic sclerosis in a French population. *J Rheumatol,* 37**,** 351-8.

GRAVES, P. N., WEISS, I. K., PERLISH, J. S. & FLEISCHMAJER, R. 1983. Increased procollagen mRNA levels in scleroderma skin fibroblasts. *J Invest Dermatol,* 80**,** 130-2.

GRESSNER, A. M., YAGMUR, E., LAHME, B., GRESSNER, O. & STANZEL, S. 2006. Connective tissue growth factor in serum as a new candidate test for assessment of hepatic fibrosis. *Clin Chem,* 52**,** 1815-7.

GRON, B., STOLTZE, K., ANDERSSON, A. & DABELSTEEN, E. 2002. Oral fibroblasts produce more HGF and KGF than skin fibroblasts in response to co-culture with keratinocytes. *APMIS,* 110**,** 892-8.

GRONE, A. 2002. Keratinocytes and cytokines. *Vet Immunol Immunopathol,* 88**,** 1-12.

GROSE, R., HUTTER, C., BLOCH, W., THOREY, I., WATT, F. M., FASSLER, R., BRAKEBUSCH, C. & WERNER, S. 2002. A crucial role of beta 1 integrins for keratinocyte migration in vitro and during cutaneous wound repair. *Development,* 129**,** 2303-15.

GROSSMAN, R. M., KRUEGER, J., YOURISH, D., GRANELLI-PIPERNO, A., MURPHY, D. P., MAY, L. T., KUPPER, T. S., SEHGAL, P. B. & GOTTLIEB, A. B. 1989. Interleukin 6 is expressed in high levels in psoriatic skin and stimulates proliferation of cultured human keratinocytes. *Proc Natl Acad Sci U S A,* 86**,** 6367-71.

GROTENDORST, G. R. & DUNCAN, M. R. 2005. Individual domains of connective tissue growth factor regulate fibroblast proliferation and myofibroblast differentiation. *FASEB J,* 19**,** 729-38.

GROTENDORST, G. R., RAHMANIE, H. & DUNCAN, M. R. 2004. Combinatorial signaling pathways determine fibroblast proliferation and myofibroblast differentiation. *FASEB J,* 18**,** 469-79.

GRUSCHWITZ, M., MULLER, P. U., SEPP, N., HOFER, E., FONTANA, A. & WICK, G. 1990. Transcription and expression of transforming growth factor type beta in the skin of progressive systemic sclerosis: a mediator of fibrosis? *J Invest Dermatol,* 94**,** 197-203.

GUARINO, M., TOSONI, A. & NEBULONI, M. 2009. Direct contribution of epithelium to organ fibrosis: epithelial-mesenchymal transition. *Hum.Pathol.,* 40**,** 1365-1376.

GUIGNARD, F., MAUEL, J. & MARKERT, M. 1996. Phosphorylation of myeloid-related proteins MRP-14 and MRP-8 during human neutrophil activation. *Eur J Biochem,* 241**,** 265-71.

GURTNER, G. C., WERNER, S., BARRANDON, Y. & LONGAKER, M. T. 2008. Wound repair and regeneration. *Nature,* 453**,** 314-21.

HAMOEN, K. E. & MORGAN, J. R. 2002. Transient Hyperproliferation of a Transgenic Human Epidermis Expressing Hepatocyte Growth Factor. *Cell Transplantation,* 11**,** 385-395.

HARDAS, B. D., ZHAO, X., ZHANG, J., LONGQING, X., STOLL, S. & ELDER, J. T. 1996. Assignment of psoriasin to human chromosomal band 1q21: coordinate overexpression of clustered genes in psoriasis. *J Invest Dermatol,* 106**,** 753-8.

HARDER, J. & NUNEZ, G. 2009. Functional expression of the intracellular pattern recognition receptor NOD1 in human keratinocytes. *J Invest Dermatol,* 129**,** 1299-302.

HASEGAWA, M., SATO, S. & TAKEHARA, K. 1999. Augmented production of chemokines (monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1alpha (MIP-1alpha) and MIP-1beta) in patients with systemic sclerosis: MCP-1 and MIP-1alpha may be involved in the development of pulmonary fibrosis. *Clin.Exp.Immunol.,* 117**,** 159-165.

HATA, R., AKAI, J., KIMURA, A., ISHIKAWA, O., KUWANA, M. & SHINKAI, H. 2000. Association of functional microsatellites in the human type I collagen alpha2 chain (COL1A2) gene with systemic sclerosis. *Biochem Biophys Res Commun,* 272**,** 36-40.

HAYASHI, N., KIDO, J., KIDO, R., WADA, C., KATAOKA, M., SHINOHARA, Y. & NAGATA, T. 2007. Regulation of calprotectin expression by interleukin-1alpha and transforming growth factor-beta in human gingival keratinocytes. *J Periodontal Res,* 42**,** 1-7.

HELL-POURMOJIB, M., NEUNER, P., FISCHER, H., REZAIE, S., KINDAS-MUGGE, I., KNOBLER, R. & TRAUTINGER, F. 2002. Differential expression of a novel gene in response to hsp27 and cell differentiation in human keratinocytes. *J Invest Dermatol,* 119**,** 154-9.

HENKE, M. O., RENNER, A., RUBIN, B. K., GYVES, J. I., LORENZ, E. & KOO, J. S. 2006. Up-regulation of S100A8 and S100A9 protein in bronchial epithelial cells by lipopolysaccharide. *Exp Lung Res,* 32**,** 331-47.

HENKEL, G. W., MCKERCHER, S. R. & MAKI, R. A. 2002. Identification of three genes up-regulated in PU.1 rescued monocytic precursor cells. *Int Immunol,* 14**,** 723-32.

HERFS, M., HUBERT, P., KHOLOD, N., CABERG, J. H., GILLES, C., BERX, G., SAVAGNER, P., BONIVER, J. & DELVENNE, P. 2008. Transforming growth factor-beta1-mediated Slug and Snail transcription factor up-regulation reduces the density of Langerhans cells in epithelial metaplasia by affecting E-cadherin expression. *Am J Pathol,* 172**,** 1391-402.

HERTIG, A., ANGLICHEAU, D., VERINE, J., PALLET, N., TOUZOT, M., ANCEL, P. Y., MESNARD, L., BROUSSE, N., BAUGEY, E., GLOTZ, D., LEGENDRE, C., RONDEAU, E. & XU-DUBOIS, Y. C. 2008. Early epithelial phenotypic changes predict graft fibrosis. *J Am Soc Nephrol,* 19**,** 1584-91.

HERTLE, M. D., KUBLER, M. D., LEIGH, I. M. & WATT, F. M. 1992. Aberrant integrin expression during epidermal wound healing and in psoriatic epidermis. *J Clin Invest,* 89**,** 1892-901.

HESSIAN, P. A., EDGEWORTH, J. & HOGG, N. 1993. MRP-8 and MRP-14, two abundant Ca(2+)-binding proteins of neutrophils and monocytes. *J Leukoc Biol,* 53**,** 197-204.

HIGASHI, K., INAGAKI, Y., FUJIMORI, K., NAKAO, A., KANEKO, H. & NAKATSUKA, I. 2003. Interferon-gamma interferes with transforming growth factor-beta signaling through direct interaction of YB-1 with Smad3. *J Biol Chem,* 278**,** 43470-9.

HIGGINS, G. C., WU, Y. & POSTLETHWAITE, A. E. 1999. Intracellular IL-1 receptor antagonist is elevated in human dermal fibroblasts that overexpress intracellular precursor IL-1 alpha. *J Immunol,* 163**,** 3969-75.

HIGLEY, H., PERSICHITTE, K., CHU, S., WAEGELL, W., VANCHEESWARAN, R. & BLACK, C. 1994. Immunocytochemical localization and serologic detection of transforming growth factor beta 1. Association with type I procollagen and inflammatory cell markers in diffuse and limited systemic sclerosis, morphea, and Raynaud's phenomenon. *Arthritis Rheum,* 37**,** 278-88.

HINZ, B., PHAN, S. H., THANNICKAL, V. J., GALLI, A., BOCHATON-PIALLAT, M. L. & GABBIANI, G. 2007. The myofibroblast: one function, multiple origins. *Am J Pathol,* 170**,** 1807-16.

HIRANO, S., REES, R. S. & GILMONT, R. R. 2002. MAP kinase pathways involving hsp27 regulate fibroblast-mediated wound contraction. *J Surg Res,* 102**,** 77-84.

HIRANO, S., SHELDEN, E. A. & GILMONT, R. R. 2004. HSP27 regulates fibroblast adhesion, motility, and matrix contraction. *Cell Stress Chaperones,* 9**,** 29-37.

HIRATSUKA, S., WATANABE, A., ABURATANI, H. & MARU, Y. 2006. Tumour-mediated upregulation of chemoattractants and recruitment of myeloid cells predetermines lung metastasis. *Nat Cell Biol,* 8**,** 1369-75.

HOCHBERG, Y. & BENJAMINI, Y. 1990. More powerful procedures for multiple significance testing. *Stat Med,* 9**,** 811-8.

HOGABOAM, C. M., STEINHAUSER, M. L., CHENSUE, S. W. & KUNKEL, S. L. 1998. Novel roles for chemokines and fibroblasts in interstitial fibrosis. *Kidney Int,* 54**,** 2152-9.

HOLMES, A., ABRAHAM, D. J., CHEN, Y., DENTON, C., SHI-WEN, X., BLACK, C. M. & LEASK, A. 2003. Constitutive connective tissue growth factor expression in scleroderma fibroblasts is dependent on Sp1. *J Biol Chem,* 278**,** 41728-33.

HOLMES, A., ABRAHAM, D. J., SA, S., SHIWEN, X., BLACK, C. M. & LEASK, A. 2001. CTGF and SMADs, maintenance of scleroderma phenotype is independent of SMAD signaling. *J Biol Chem,* 276**,** 10594-601.

HONDA, N., JINNIN, M., KAJIHARA, I., MAKINO, T., MAKINO, K., MASUGUCHI, S., FUKUSHIMA, S., OKAMOTO, Y., HASEGAWA, M., FUJIMOTO, M. & IHN, H. 2012. TGF-beta-mediated downregulation of microRNA-196a contributes to the constitutive upregulated type I collagen expression in scleroderma dermal fibroblasts. *J Immunol,* 188**,** 3323-31.

HOOT, K. E., LIGHTHALL, J., HAN, G., LU, S. L., LI, A., JU, W., KULESZ-MARTIN, M., BOTTINGER, E. & WANG, X. J. 2008. Keratinocyte-specific Smad2 ablation results in increased epithelial-mesenchymal transition during skin cancer formation and progression. *J.Clin.Invest,* 118**,** 2722-2732.

HORLEIN, D., MCPHERSON, J., GOH, S. H. & BORNSTEIN, P. 1981. Regulation of protein synthesis: translational control by procollagen-derived fragments. *Proc Natl Acad Sci U S A,* 78**,** 6163-7.

HUBER, M., RETTLER, I., BERNASCONI, K., FRENK, E., LAVRIJSEN, S. P., PONEC, M., BON, A., LAUTENSCHLAGER, S., SCHORDERET, D. F. & HOHL, D. 1995. Mutations of keratinocyte transglutaminase in lamellar ichthyosis. *Science,* 267**,** 525-8.

HUBNER, G., ALZHEIMER, C. & WERNER, S. 1999. Activin: a novel player in tissue repair processes. *Histol Histopathol,* 14**,** 295-304.

HUDSON, L. L., ROCCA, K. M., KUWANA, M. & PANDEY, J. P. 2005. Interleukin-10 genotypes are associated with systemic sclerosis and influence disease-associated autoimmune responses. *Genes Immun,* 6**,** 274-8.

HUMMERS, L. K., HALL, A., WIGLEY, F. M. & SIMONS, M. 2009. Abnormalities in the regulators of angiogenesis in patients with scleroderma. *J Rheumatol,* 36**,** 576-82.

HUMPHREYS, B. D., LIN, S. L., KOBAYASHI, A., HUDSON, T. E., NOWLIN, B. T., BONVENTRE, J. V., VALERIUS, M. T., MCMAHON, A. P. & DUFFIELD, J. S. 2010. Fate tracing reveals the pericyte and not epithelial origin of myofibroblasts in kidney fibrosis. *Am.J.Pathol.,* 176**,** 85-97.

HYNES, R. O. 2009. The extracellular matrix: not just pretty fibrils. *Science,* 326**,** 1216-9.

IGARASHI, A., NASHIRO, K., KIKUCHI, K., SATO, S., IHN, H., FUJIMOTO, M., GROTENDORST, G. R. & TAKEHARA, K. 1996. Connective tissue growth factor gene expression in tissue sections from localized scleroderma, keloid, and other fibrotic skin disorders. *J Invest Dermatol,* 106**,** 729-33.

IHN, H. 2005. Scleroderma, fibroblasts, signaling, and excessive extracellular matrix. *Curr Rheumatol Rep,* 7**,** 156-62.

IHN, H., YAMANE, K., ASANO, Y., JINNIN, M. & TAMAKI, K. 2006. Constitutively phosphorylated Smad3 interacts with Sp1 and p300 in scleroderma fibroblasts. *Rheumatology (Oxford),* 45**,** 157-65.

ISAKSEN, B. & FAGERHOL, M. K. 2001. Calprotectin inhibits matrix metalloproteinases by sequestration of zinc. *Mol Pathol,* 54**,** 289-92.

ISHIDA-YAMAMOTO, A., MCGRATH, J. A., LAM, H., IIZUKA, H., FRIEDMAN, R. A. & CHRISTIANO, A. M. 1997. The molecular pathology of progressive symmetric

erythrokeratoderma: a frameshift mutation in the loricrin gene and perturbations in the cornified cell envelope. *Am J Hum Genet,* 61**,** 581-9.

ITOU, H., YAO, M., FUJITA, I., WATANABE, N., SUZUKI, M., NISHIHIRA, J. & TANAKA, I. 2002. The crystal structure of human MRP14 (S100A9), a Ca(2+)-dependent regulator protein in inflammatory process. *J Mol Biol,* 316**,** 265-76.

IVKOVIC, S., YOON, B. S., POPOFF, S. N., SAFADI, F. F., LIBUDA, D. E., STEPHENSON, R. C., DALUISKI, A. & LYONS, K. M. 2003. Connective tissue growth factor coordinates chondrogenesis and angiogenesis during skeletal development. *Development,* 130**,** 2779-91.

IWANO, M., PLIETH, D., DANOFF, T. M., XUE, C., OKADA, H. & NEILSON, E. G. 2002. Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J Clin Invest,* 110**,** 341-50.

JAIN, M., RIVERA, S., MONCLUS, E. A., SYNENKI, L., ZIRK, A., EISENBART, J., FEGHALI-BOSTWICK, C., MUTLU, G. M., BUDINGER, G. R. & CHANDEL, N. S. 2013. Mitochondrial reactive oxygen species regulate transforming growth factor-beta signaling. *J Biol Chem,* 288**,** 770-7.

JAVELAUD, D. & MAUVIEL, A. 2004. Mammalian transforming growth factor-betas: Smad signaling and physio-pathological roles. *Int J Biochem Cell Biol,* 36**,** 1161-5.

JENSEN, L. J., KUHN, M., STARK, M., CHAFFRON, S., CREEVEY, C., MULLER, J., DOERKS, T., JULIEN, P., ROTH, A., SIMONOVIC, M., BORK, P. & VON MERING, C. 2009. STRING 8--a global view on proteins and their functional interactions in 630 organisms. *Nucleic Acids Res,* 37**,** D412-6.

JIMENEZ, S. A. & BASHEY, R. I. 1977. Collagen synthesis by scleroderma fibroblasts in culture. *Arthritis Rheum,* 20**,** 902-3.

JIMENEZ, S. A., FELDMAN, G., BASHEY, R. I., BIENKOWSKI, R. & ROSENBLOOM, J. 1986. Co-ordinate increase in the expression of type I and type III collagen genes in progressive systemic sclerosis fibroblasts. *Biochem J,* 237**,** 837-43.

JINNIN, M., IHN, H., MIMURA, Y., ASANO, Y., YAMANE, K. & TAMAKI, K. 2005. Effects of hepatocyte growth factor on the expression of type I collagen and matrix metalloproteinase-1 in normal and scleroderma dermal fibroblasts. *J Invest Dermatol,* 124**,** 324-30.

JONAK, C., MILDNER, M., KLOSNER, G., PAULITSCHKE, V., KUNSTFELD, R., PEHAMBERGER, H., TSCHACHLER, E. & TRAUTINGER, F. 2011. The hsp27kD heat shock protein and p38-MAPK signaling are required for regular epidermal differentiation. *J Dermatol Sci,* 61**,** 32-7.

JONES, P. H. & WATT, F. M. 1993. Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. *Cell,* 73**,** 713-24.

KAARTINEN, V., VONCKEN, J. W., SHULER, C., WARBURTON, D., BU, D., HEISTERKAMP, N. & GROFFEN, J. 1995. Abnormal lung development and cleft palate in mice lacking TGF-beta 3 indicates defects of epithelial-mesenchymal interaction. *Nat Genet,* 11**,** 415-21.

KADLER, K. E., BALDOCK, C., BELLA, J. & BOOT-HANDFORD, R. P. 2007. Collagens at a glance. *J Cell Sci,* 120**,** 1955-8.

KADONO, T., KIKUCHI, K., IHN, H., TAKEHARA, K. & TAMAKI, K. 1998. Increased production of interleukin 6 and interleukin 8 in scleroderma fibroblasts. *J Rheumatol,* 25**,** 296-301.

KADONO, T., KIKUCHI, K., KUBO, M., FUJIMOTO, M. & TAMAKI, K. 1996. Serum concentrations of basic fibroblast growth factor in collagen diseases. *J Am Acad Dermatol,* 35**,** 392-7.

KAHALEH, M. B. & LEROY, E. C. 1999. Autoimmunity and vascular involvement in systemic sclerosis (SSc). *Autoimmunity,* 31**,** 195-214.

KAHAN, A., COGHLAN, G. & MCLAUGHLIN, V. 2009. Cardiac complications of systemic sclerosis. *Rheumatology (Oxford),* 48 Suppl 3**,** iii45-8.

KALALI, B. N., KOLLISCH, G., MAGES, J., MULLER, T., BAUER, S., WAGNER, H., RING, J., LANG, R., MEMPEL, M. & OLLERT, M. 2008. Double-stranded RNA induces an antiviral defense status in epidermal keratinocytes through TLR3-, PKR-, and MDA5/RIG-I-mediated differential signaling. *J Immunol,* 181**,** 2694-704.

KALLBERG, E., VOGL, T., LIBERG, D., OLSSON, A., BJORK, P., WIKSTROM, P., BERGH, A., ROTH, J., IVARS, F. & LEANDERSON, T. 2012. S100A9 interaction with TLR4 promotes tumor growth. *PLoS One,* 7**,** e34207.

KALLURI, R. & NEILSON, E. G. 2003. Epithelial-mesenchymal transition and its implications for fibrosis. *J.Clin.Invest,* 112**,** 1776-1784.

KALLURI, R. & WEINBERG, R. A. 2009. The basics of epithelial-mesenchymal transition. *J Clin Invest,* 119**,** 1420-8.

KANANGAT, S., POSTLETHWAITE, A. E., HIGGINS, G. C. & HASTY, K. A. 2006. Novel functions of intracellular IL-1ra in human dermal fibroblasts: implications in the pathogenesis of fibrosis. *J Invest Dermatol,* 126**,** 756-65.

KANE, C. J., HEBDA, P. A., MANSBRIDGE, J. N. & HANAWALT, P. C. 1991. Direct evidence for spatial and temporal regulation of transforming growth factor beta 1 expression during cutaneous wound healing. *J Cell Physiol,* 148**,** 157-73.

KARAKECILI, A. G., SATRIANO, C., GUMUSDERELIOGLU, M. & MARLETTA, G. 2008. Enhancement of fibroblastic proliferation on chitosan surfaces by immobilized epidermal growth factor. *Acta Biomater,* 4**,** 989-96.

KARRER, S., BOSSERHOFF, A. K., WEIDERER, P., DISTLER, O., LANDTHALER, M., SZEIMIES, R. M., MULLER-LADNER, U., SCHOLMERICH, J. & HELLERBRAND, C. 2005. The -2518 promotor polymorphism in the MCP-1 gene is associated with systemic sclerosis. *J Invest Dermatol,* 124**,** 92-8.

KAWAGUCHI, Y. 1994. IL-1 alpha gene expression and protein production by fibroblasts from patients with systemic sclerosis. *Clin Exp Immunol,* 97**,** 445-50.

KAWAGUCHI, Y., HARA, M. & WRIGHT, T. M. 1999. Endogenous IL-1alpha from systemic sclerosis fibroblasts induces IL-6 and PDGF-A. *J Clin Invest,* 103**,** 1253-60.

KAWAGUCHI, Y., HARIGAI, M., HARA, M., FUKASAWA, C., TAKAGI, K., TANAKA, M., TANAKA, E., NISHIMAGI, E. & KAMATANI, N. 2002. Expression of hepatocyte growth factor and its receptor (c-met) in skin fibroblasts from patients with systemic sclerosis. *J Rheumatol,* 29**,** 1877-83.

KAWAGUCHI, Y., HARIGAI, M., SUZUKI, K., HARA, M., KOBAYASHI, K., ISHIZUKA, T., MATSUKI, Y., TANAKA, N. & NAKAMURA, H. 1993. Interleukin 1 receptor on fibroblasts from systemic sclerosis patients induces excessive functional responses to interleukin 1 beta. *Biochem Biophys Res Commun,* 190**,** 154-61.

KAWAGUCHI, Y., NISHIMAGI, E., TOCHIMOTO, A., KAWAMOTO, M., KATSUMATA, Y., SOEJIMA, M., KANNO, T., KAMATANI, N. & HARA, M. 2006. Intracellular IL-1alpha-binding proteins contribute to biological functions of endogenous IL-

1alpha in systemic sclerosis fibroblasts. *Proc Natl Acad Sci U S A,* 103**,** 14501-6.

KAWAGUCHI, Y., OTA, Y., KAWAMOTO, M., ITO, I., TSUCHIYA, N., SUGIURA, T., KATSUMATA, Y., SOEJIMA, M., SATO, S., HASEGAWA, M., FUJIMOTO, M., TAKEHARA, K., KUWANA, M., YAMANAKA, H. & HARA, M. 2009. Association study of a polymorphism of the CTGF gene and susceptibility to systemic sclerosis in the Japanese population. *Ann Rheum Dis,* 68**,** 1921-4.

KAWAGUCHI, Y., TOCHIMOTO, A., ICHIKAWA, N., HARIGAI, M., HARA, M., KOTAKE, S., KITAMURA, Y. & KAMATANI, N. 2003. Association of IL1A gene polymorphisms with susceptibility to and severity of systemic sclerosis in the Japanese population. *Arthritis Rheum,* 48**,** 186-92.

KAWAI, H., MINAMIYA, Y. & TAKAHASHI, N. 2011. Prognostic impact of S100A9 overexpression in non-small cell lung cancer. *Tumour Biol,* 32**,** 641-6.

KEMP, T. J., AGGELI, I. K., SUGDEN, P. H. & CLERK, A. 2004. Phenylephrine and endothelin-1 upregulate connective tissue growth factor in neonatal rat cardiac myocytes. *J Mol Cell Cardiol,* 37**,** 603-6.

KERKHOFF, C., HOFMANN, H. A., VORMOOR, J., MELKONYAN, H., ROTH, J., SORG, C. & KLEMPT, M. 2002. Binding of two nuclear complexes to a novel regulatory element within the human S100A9 promoter drives the S100A9 gene expression. *J Biol Chem,* 277**,** 41879-87.

KERKHOFF, C., SORG, C., TANDON, N. N. & NACKEN, W. 2001. Interaction of S100A8/S100A9-arachidonic acid complexes with the scavenger receptor CD36 may facilitate fatty acid uptake by endothelial cells. *Biochemistry,* 40**,** 241-8.

KHAN, K., XU, S., NIHTYANOVA, S., DERRETT-SMITH, E., ABRAHAM, D., DENTON, C. P. & ONG, V. H. 2012. Clinical and pathological significance of interleukin 6 overexpression in systemic sclerosis. *Ann Rheum Dis,* 71**,** 1235-42.

KIKUCHI, K., IHN, H., SATO, S., IGARASHI, A., SOMA, Y., ISHIBASHI, Y. & TAKEHARA, K. 1994. Serum concentration of procollagen type I carboxyterminal propeptide in systemic sclerosis. *Arch Dermatol Res,* 286**,** 77-80.

KIM, K. K., KUGLER, M. C., WOLTERS, P. J., ROBILLARD, L., GALVEZ, M. G., BRUMWELL, A. N., SHEPPARD, D. & CHAPMAN, H. A. 2006. Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and

is regulated by the extracellular matrix. *Proc Natl Acad Sci U S A,* 103**,** 13180-5.

KISICH, K. O., HOWELL, M. D., BOGUNIEWICZ, M., HEIZER, H. R., WATSON, N. U. & LEUNG, D. Y. 2007. The constitutive capacity of human keratinocytes to kill Staphylococcus aureus is dependent on beta-defensin 3. *J Invest Dermatol,* 127**,** 2368-80.

KLARESKOG, L., GUSTAFSSON, R., SCHEYNIUS, A. & HALLGREN, R. 1990. Increased expression of platelet-derived growth factor type B receptors in the skin of patients with systemic sclerosis. *Arthritis Rheum,* 33**,** 1534-41.

KLEMPT, M., MELKONYAN, H., HOFMANN, H. A., EUE, I. & SORG, C. 1998. The transcription factors c-myb and C/EBP alpha regulate the monocytic/myeloic gene MRP14. *Immunobiology,* 199**,** 148-51.

KOBAYASHI, M., YOSHIKI, R., SAKABE, J., KABASHIMA, K., NAKAMURA, M. & TOKURA, Y. 2009. Expression of toll-like receptor 2, NOD2 and dectin-1 and stimulatory effects of their ligands and histamine in normal human keratinocytes. *Br J Dermatol,* 160**,** 297-304.

KOCH, A. E., KRONFELD-HARRINGTON, L. B., SZEKANECZ, Z., CHO, M. M., HAINES, G. K., HARLOW, L. A., STRIETER, R. M., KUNKEL, S. L., MASSA, M. C., BARR, W. G. & ET AL. 1993. In situ expression of cytokines and cellular adhesion molecules in the skin of patients with systemic sclerosis. Their role in early and late disease. *Pathobiology,* 61**,** 239-46.

KOESTERS, R., KAISSLING, B., LEHIR, M., PICARD, N., THEILIG, F., GEBHARDT, R., GLICK, A. B., HAHNEL, B., HOSSER, H., GRONE, H. J. & KRIZ, W. 2010. Tubular overexpression of transforming growth factor-beta1 induces autophagy and fibrosis but not mesenchymal transition of renal epithelial cells. *Am J Pathol,* 177**,** 632-43.

KOLLS, J. K., MCCRAY, P. B., JR. & CHAN, Y. R. 2008. Cytokine-mediated regulation of antimicrobial proteins. *Nat Rev Immunol,* 8**,** 829-35.

KONDO, K., OKADA, T., MATSUI, T., KATO, S., DATE, K., YOSHIHARA, M., NAGATA, Y., TAKAGI, H., YONEDA, M. & SUGIE, I. 2001. Establishment and characterization of a human B cell line from the lung tissue of a patient with scleroderma; extraordinary high level of IL-6 secretion by stimulated fibroblasts. *Cytokine,* 13**,** 220-6.

KOOL, J., REUBSAET, L., WESSELDIJK, F., MARAVILHA, R. T., PINKSE, M. W., D'SANTOS, C. S., VAN HILTEN, J. J., ZIJLSTRA, F. J. & HECK, A. J. 2007. Suction blister fluid as potential body fluid for biomarker proteins. *Proteomics,* 7**,** 3638-50.

KORNDORFER, I. P., BRUECKNER, F. & SKERRA, A. 2007. The crystal structure of the human (S100A8/S100A9)2 heterotetramer, calprotectin, illustrates how conformational changes of interacting alpha-helices can determine specific association of two EF-hand proteins. *J Mol Biol,* 370**,** 887-98.

KOSKELA, A., ENGSTROM, K., HAKELIUS, M., NOWINSKI, D. & IVARSSON, M. 2010. Regulation of fibroblast gene expression by keratinocytes in organotypic skin culture provides possible mechanisms for the antifibrotic effect of reepithelialization. *Wound Repair Regen,* 18**,** 452-9.

KOVALENKO, E., TACKE, F., GRESSNER, O. A., ZIMMERMANN, H. W., LAHME, B., JANETZKO, A., WIEDERHOLT, T., BERG, T., MULLER, T., TRAUTWEIN, C., GRESSNER, A. M. & WEISKIRCHEN, R. 2009. Validation of connective tissue growth factor (CTGF/CCN2) and its gene polymorphisms as noninvasive biomarkers for the assessment of liver fibrosis. *J Viral Hepat,* 16**,** 612-20.

KRETSCHMER, A., MOEPERT, K., DAMES, S., STERNBERGER, M., KAUFMANN, J. & KLIPPEL, A. 2003. Differential regulation of TGF-beta signaling through Smad2, Smad3 and Smad4. *Oncogene,* 22**,** 6748-63.

KRIEG, T., ABRAHAM, D. & LAFYATIS, R. 2007. Fibrosis in connective tissue disease: the role of the myofibroblast and fibroblast-epithelial cell interactions. *Arthritis Res Ther,* 9 Suppl 2**,** S4.

KRIEG, T., HORLEIN, D., WIESTNER, M. & MULLER, P. K. 1978. Aminoterminal extension peptides from type I procollagen normalize excessive collagen synthesis of scleroderma fibroblasts. *Arch Dermatol Res,* 263**,** 171-80.

KUHN, C. & MCDONALD, J. A. 1991. The roles of the myofibroblast in idiopathic pulmonary fibrosis. Ultrastructural and immunohistochemical features of sites of active extracellular matrix synthesis. *Am J Pathol,* 138**,** 1257-65.

KURUTO, R., NOZAWA, R., TAKEISHI, K., ARAI, K., YOKOTA, T. & TAKASAKI, Y. 1990. Myeloid Calcium Binding Proteins: Expression in the Differentiated HL-60 Cells and Detection in Sera of Patients with Connective Tissue Diseases. *Journal of Biochemistry,* 108**,** 650-653.

KURUTO-NIWA, R., NAKAMURA, M., TAKEISHI, K. & NOZAWA, R. 1998. Transcriptional regulation by C/EBP alpha and -beta in the expression of the gene for the MRP14 myeloid calcium binding protein. *Cell Struct Funct,* 23**,** 109-18.

LAI, Y. & GALLO, R. L. 2009. AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense. *Trends Immunol,* 30**,** 131-41.

LAMOUILLE, S. & DERYNCK, R. 2007. Cell size and invasion in TGF-beta-induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway. *J Cell Biol,* 178**,** 437-51.

LASKY, J. A., ORTIZ, L. A., TONTHAT, B., HOYLE, G. W., CORTI, M., ATHAS, G., LUNGARELLA, G., BRODY, A. & FRIEDMAN, M. 1998. Connective tissue growth factor mRNA expression is upregulated in bleomycin-induced lung fibrosis. *Am J Physiol,* 275**,** L365-71.

LAWRENCE, A., KHANNA, D., MISRA, R. & AGGARWAL, A. 2006. Increased expression of basic fibroblast growth factor in skin of patients with systemic sclerosis. *Dermatol.Online.J.,* 12**,** 2.

LEASK, A. & ABRAHAM, D. J. 2006. All in the CCN family: essential matricellular signaling modulators emerge from the bunker. *J Cell Sci,* 119**,** 4803-10.

LEASK, A., ABRAHAM, D. J., FINLAY, D. R., HOLMES, A., PENNINGTON, D., SHI-WEN, X., CHEN, Y., VENSTROM, K., DOU, X., PONTICOS, M., BLACK, C., BERNABEU, C., JACKMAN, J. K., FINDELL, P. R. & CONNOLLY, M. K. 2002. Dysregulation of transforming growth factor beta signaling in scleroderma: overexpression of endoglin in cutaneous scleroderma fibroblasts. *Arthritis Rheum,* 46**,** 1857-65.

LEBLEU, V. S., TADURI, G., O'CONNELL, J., TENG, Y., COOKE, V. G., WODA, C., SUGIMOTO, H. & KALLURI, R. 2013. Origin and function of myofibroblasts in kidney fibrosis. *Nat Med,* 19**,** 1047-53.

LEBRE, M. C., VAN DER AAR, A. M., VAN BAARSEN, L., VAN CAPEL, T. M., SCHUITEMAKER, J. H., KAPSENBERG, M. L. & DE JONG, E. C. 2007. Human keratinocytes express functional Toll-like receptor 3, 4, 5, and 9. *J Invest Dermatol,* 127**,** 331-41.

LEE, M. J., LEE, J.-K., CHOI, J. W., LEE, C.-S., SIM, J. H., CHO, C.-H., LEE, K.-H., CHO, I.-H., CHUNG, M.-H., KIM, H.-R. & YE, S.-K. 2012. Interleukin-6 Induces S100A9

Expression in Colonic Epithelial Cells through STAT3 Activation in Experimental Ulcerative Colitis. *PLoS One,* 7**,** e38801.

LEROY, E. C. 1974. Increased collagen synthesis by scleroderma skin fibroblasts in vitro: a possible defect in the regulation or activation of the scleroderma fibroblast. *J Clin Invest,* 54**,** 880-9.

LEROY, E. C., BLACK, C., FLEISCHMAJER, R., JABLONSKA, S., KRIEG, T., MEDSGER, T. A., JR., ROWELL, N. & WOLLHEIM, F. 1988. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. *J Rheumatol,* 15**,** 202-5.

LEUNG, L. Y., TIAN, D., BRANGWYNNE, C. P., WEITZ, D. A. & TSCHUMPERLIN, D. J. 2007. A new microrheometric approach reveals individual and cooperative roles for TGF-beta1 and IL-1beta in fibroblast-mediated stiffening of collagen gels. *FASEB J,* 21**,** 2064-73.

LI, E. R., OWENS, D. M., DJIAN, P. & WATT, F. M. 2000. Expression of involucrin in normal, hyperproliferative and neoplastic mouse keratinocytes. *Exp Dermatol,* 9**,** 431-8.

LI, G., XIE, Q., SHI, Y., LI, D., ZHANG, M., JIANG, S., ZHOU, H., LU, H. & JIN, Y. 2006. Inhibition of connective tissue growth factor by siRNA prevents liver fibrosis in rats. *J Gene Med,* 8**,** 889-900.

LIAKATH-ALI, K., VANCOLLIE, V. E., HEATH, E., SMEDLEY, D. P., ESTABEL, J., SUNTER, D., DITOMMASO, T., WHITE, J. K., RAMIREZ-SOLIS, R., SMYTH, I., STEEL, K. P. & WATT, F. M. 2014. Novel skin phenotypes revealed by a genome-wide mouse reverse genetic screen. *Nat Commun,* 5**,** 3540.

LIANG, S. C., TAN, X. Y., LUXENBERG, D. P., KARIM, R., DUNUSSI-JOANNOPOULOS, K., COLLINS, M. & FOUSER, L. A. 2006. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med,* 203**,** 2271-9.

LIAO, H., WU, J., KUHN, E., CHIN, W., CHANG, B., JONES, M. D., O'NEIL, S., CLAUSER, K. R., KARL, J., HASLER, F., ROUBENOFF, R., ZOLG, W. & GUILD, B. C. 2004. Use of mass spectrometry to identify protein biomarkers of disease severity in the synovial fluid and serum of patients with rheumatoid arthritis. *Arthritis and Rheumatism,* 50**,** 3792-3803.

LINDAHL, G. E., CHAMBERS, R. C., PAPAKRIVOPOULOU, J., DAWSON, S. J., JACOBSEN, M. C., BISHOP, J. E. & LAURENT, G. J. 2002. Activation of fibroblast procollagen

alpha 1(I) transcription by mechanical strain is transforming growth factor-beta-dependent and involves increased binding of CCAAT-binding factor (CBF/NF-Y) at the proximal promoter. *J Biol Chem,* 277**,** 6153-61.

LIU, S., TAGHAVI, R. & LEASK, A. 2010. Connective tissue growth factor is induced in bleomycin-induced skin scleroderma. *J Cell Commun Signal,* 4**,** 25-30.

LIVAK, K. J. & SCHMITTGEN, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods,* 25**,** 402-8.

LLOYD, C. M., DORF, M. E., PROUDFOOT, A., SALANT, D. J. & GUTIERREZ-RAMOS, J. C. 1997a. Role of MCP-1 and RANTES in inflammation and progression to fibrosis during murine crescentic nephritis. *J Leukoc Biol,* 62**,** 676-80.

LLOYD, C. M., MINTO, A. W., DORF, M. E., PROUDFOOT, A., WELLS, T. N., SALANT, D. J. & GUTIERREZ-RAMOS, J. C. 1997b. RANTES and monocyte chemoattractant protein-1 (MCP-1) play an important role in the inflammatory phase of crescentic nephritis, but only MCP-1 is involved in crescent formation and interstitial fibrosis. *J Exp Med,* 185**,** 1371-80.

LOPEZ-NOVOA, J. M. & NIETO, M. A. 2009. Inflammation and EMT: an alliance towards organ fibrosis and cancer progression. *EMBO Mol Med,* 1**,** 303-14.

LUGER, T. A. & SCHWARZ, T. 1990. Evidence for an epidermal cytokine network. *J Invest Dermatol,* 95**,** 100S-104S.

LUGERING, N., STOLL, R., SCHMID, K. W., KUCHARZIK, T., STEIN, H., BURMEISTER, G., SORG, C. & DOMSCHKE, W. 1995. The myeloic related protein MRP8/14 (27E10 antigen)--usefulness as a potential marker for disease activity in ulcerative colitis and putative biological function. *Eur J Clin Invest,* 25**,** 659-64.

MAAS-SZABOWSKI, N. & FUSENIG, N. E. 1996. Interleukin-1-induced growth factor expression in postmitotic and resting fibroblasts. *J Invest Dermatol,* 107**,** 849-55.

MAAS-SZABOWSKI, N., STARK, H. J. & FUSENIG, N. E. 2000. Keratinocyte growth regulation in defined organotypic cultures through IL-1-induced keratinocyte growth factor expression in resting fibroblasts. *J Invest Dermatol,* 114**,** 1075-84.

MAEDA, M., MATUBARA, K., HIRANO, H., WATABE, H., ICHIKI, Y. & MORI, S. 1993. Pitting scars in progressive systemic sclerosis. *Dermatology,* 187**,** 104-8.

MAESTRINI, E., MONACO, A. P., MCGRATH, J. A., ISHIDA-YAMAMOTO, A., CAMISA, C., HOVNANIAN, A., WEEKS, D. E., LATHROP, M., UITTO, J. & CHRISTIANO, A. M. 1996. A molecular defect in loricrin, the major component of the cornified cell envelope, underlies Vohwinkel's syndrome. *Nat Genet,* 13**,** 70-7.

MAIZELS, E. T., PETERS, C. A., KLINE, M., CUTLER, R. E., JR., SHANMUGAM, M. & HUNZICKER-DUNN, M. 1998. Heat-shock protein-25/27 phosphorylation by the delta isoform of protein kinase C. *Biochem J,* 332 ( Pt 3)**,** 703-12.

MAJEWSKI, S., HUNZELMANN, N., JOHNSON, J. P., JUNG, C., MAUCH, C., ZIEGLER-HEITBROCK, H. W., RIETHMULLER, G. & KRIEG, T. 1991. Expression of intercellular adhesion molecule-1 (ICAM-1) in the skin of patients with systemic scleroderma. *J Invest Dermatol,* 97**,** 667-71.

MAKOL, A., REILLY, M. J. & ROSENMAN, K. D. 2011. Prevalence of connective tissue disease in silicosis (1985-2006)-a report from the state of Michigan surveillance system for silicosis. *Am J Ind Med,* 54**,** 255-62.

MANSBRIDGE, J. N. & KNAPP, A. M. 1987. Changes in keratinocyte maturation during wound healing. *J Invest Dermatol,* 89**,** 253-63.

MARINKOVICH, M. P., KEENE, D. R., RIMBERG, C. S. & BURGESON, R. E. 1993. Cellular origin of the dermal-epidermal basement membrane. *Dev Dyn,* 197**,** 255-67.

MARIONNET, C., BERNERD, F., DUMAS, A., VERRECCHIA, F., MOLLIER, K., COMPAN, D., BERNARD, B., LAHFA, M., LECLAIRE, J., MEDAISKO, C., MEHUL, B., SEITE, S., MAUVIEL, A. & DUBERTRET, L. 2003. Modulation of gene expression induced in human epidermis by environmental stress in vivo. *J Invest Dermatol,* 121**,** 1447-58.

MARTIN, J. E., ASSASSI, S., DIAZ-GALLO, L. M., BROEN, J. C., SIMEON, C. P., CASTELLVI, I., VICENTE-RABANEDA, E., FONOLLOSA, V., ORTEGO-CENTENO, N., GONZALEZ-GAY, M. A., ESPINOSA, G., CARREIRA, P., CAMPS, M., SABIO, J. M., D'ALFONSO, S., VONK, M. C., VOSKUYL, A. E., SCHUERWEGH, A. J., KREUTER, A., WITTE, T., RIEMEKASTEN, G., HUNZELMANN, N., AIRO, P., BERETTA, L., SCORZA, R., LUNARDI, C., VAN LAAR, J., CHEE, M. M., WORTHINGTON, J., HERRICK, A., DENTON, C., FONSECA, C., TAN, F. K., ARNETT, F., ZHOU, X., REVEILLE, J. D., GORLOVA, O., KOELEMAN, B. P., RADSTAKE, T. R., VYSE, T., MAYES, M. D., ALARCON-RIQUELME, M. E. &

MARTIN, J. 2013. A systemic sclerosis and systemic lupus erythematosus pan-meta-GWAS reveals new shared susceptibility loci. *Hum Mol Genet*.

MARTIN, P., TEODORO, W. R., VELOSA, A. P., DE MORAIS, J., CARRASCO, S., CHRISTMANN, R. B., GOLDENSTEIN-SCHAINBERG, C., PARRA, E. R., KATAYAMA, M. L., SOTTO, M. N., CAPELOZZI, V. L. & YOSHINARI, N. H. 2012. Abnormal collagen V deposition in dermis correlates with skin thickening and disease activity in systemic sclerosis. *Autoimmun Rev,* 11**,** 827-35.

MARTINSSON, H., YHR, M. & ENERBACK, C. 2005. Expression patterns of S100A7 (psoriasin) and S100A9 (calgranulin-B) in keratinocyte differentiation. *Exp Dermatol,* 14**,** 161-8.

MATSUMOTO, K., HASHIMOTO, K., HASHIRO, M., YOSHIMASA, H. & YOSHIKAWA, K. 1990. Modulation of growth and differentiation in normal human keratinocytes by transforming growth factor-beta. *J Cell Physiol,* 145**,** 95-101.

MATSUMOTO, K., HASHIMOTO, K., YOSHIKAWA, K. & NAKAMURA, T. 1991. Marked stimulation of growth and motility of human keratinocytes by hepatocyte growth factor. *Exp Cell Res,* 196**,** 114-120.

MATSUNAGA, N., TSUCHIMORI, N., MATSUMOTO, T. & II, M. 2011. TAK-242 (resatorvid), a small-molecule inhibitor of Toll-like receptor (TLR) 4 signaling, binds selectively to TLR4 and interferes with interactions between TLR4 and its adaptor molecules. *Mol Pharmacol,* 79**,** 34-41.

MATUCCI-CERINIC, M., KAHALEH, B. & WIGLEY, F. M. 2013. Review: evidence that systemic sclerosis is a vascular disease. *Arthritis Rheum,* 65**,** 1953-62.

MAYES, M. D. 1997. Epidemiology of systemic sclerosis and related diseases. *Curr Opin Rheumatol,* 9**,** 557-61.

MAYES, M. D., BOSSINI-CASTILLO, L., GORLOVA, O., MARTIN, J. E., ZHOU, X., CHEN, W. V., ASSASSI, S., YING, J., TAN, F. K., ARNETT, F. C., REVEILLE, J. D., GUERRA, S., TERUEL, M., CARMONA, F. D., GREGERSEN, P. K., LEE, A. T., LOPEZ-ISAC, E., OCHOA, E., CARREIRA, P., SIMEON, C. P., CASTELLVI, I., GONZALEZ-GAY, M. A., ZHERNAKOVA, A., PADYUKOV, L., ALARCON-RIQUELME, M., WIJMENGA, C., BROWN, M., BERETTA, L., RIEMEKASTEN, G., WITTE, T., HUNZELMANN, N., KREUTER, A., DISTLER, J. H., VOSKUYL, A. E., SCHUERWEGH, A. J., HESSELSTRAND, R., NORDIN, A., AIRO, P., LUNARDI, C., SHIELS, P., VAN LAAR, J. M., HERRICK, A., WORTHINGTON, J., DENTON, C., WIGLEY, F. M., HUMMERS, L. K., VARGA, J., HINCHCLIFF, M. E., BARON, M., HUDSON, M.,

POPE, J. E., FURST, D. E., KHANNA, D., PHILLIPS, K., SCHIOPU, E., SEGAL, B. M., MOLITOR, J. A., SILVER, R. M., STEEN, V. D., SIMMS, R. W., LAFYATIS, R. A., FESSLER, B. J., FRECH, T. M., ALKASSAB, F., DOCHERTY, P., KAMINSKA, E., KHALIDI, N., JONES, H. N., MARKLAND, J., ROBINSON, D., BROEN, J., RADSTAKE, T. R., FONSECA, C., KOELEMAN, B. P. & MARTIN, J. 2014. Immunochip analysis identifies multiple susceptibility loci for systemic sclerosis. *Am J Hum Genet,* 94**,** 47-61.

MCANULTY, R. J. 2007. Fibroblasts and myofibroblasts: their source, function and role in disease. *Int J Biochem Cell Biol,* 39**,** 666-71.

MCCORMIC, Z. D., KHUDER, S. S., ARYAL, B. K., AMES, A. L. & KHUDER, S. A. 2010. Occupational silica exposure as a risk factor for scleroderma: a meta-analysis. *Int Arch Occup Environ Health,* 83**,** 763-9.

MCNEILL, E., CONWAY, S. J., RODERICK, H. L., BOOTMAN, M. D. & HOGG, N. 2007. Defective chemoattractant-induced calcium signalling in S100A9 null neutrophils. *Cell Calcium,* 41**,** 107-21.

MERAD, M., GINHOUX, F. & COLLIN, M. 2008. Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells. *Nat Rev Immunol,* 8**,** 935-47.

MEYER, M., MULLER, A. K., YANG, J., SBREVE ULCOVA, J. & WERNER, S. 2011. The role of chronic inflammation in cutaneous fibrosis: fibroblast growth factor receptor deficiency in keratinocytes as an example. *J Investig Dermatol Symp Proc,* 15**,** 48-52.

MILANO, A., PENDERGRASS, S. A., SARGENT, J. L., GEORGE, L. K., MCCALMONT, T. H., CONNOLLY, M. K. & WHITFIELD, M. L. 2008. Molecular subsets in the gene expression signatures of scleroderma skin. *PLoS.One.,* 3**,** e2696.

MISCHKE, D., KORGE, B. P., MARENHOLZ, I., VOLZ, A. & ZIEGLER, A. 1996. Genes encoding structural proteins of epidermal cornification and S100 calcium-binding proteins form a gene complex ("epidermal differentiation complex") on human chromosome 1q21. *J Invest Dermatol,* 106**,** 989-92.

MORI, Y., CHEN, S. J. & VARGA, J. 2003. Expression and regulation of intracellular SMAD signaling in scleroderma skin fibroblasts. *Arthritis Rheum,* 48**,** 1964-78.

MORLEY, S. M., GAYLARDE, P. M. & SARKANY, I. 1985a. Epidermal thickness in systemic sclerosis and morphoea. *Clin.Exp.Dermatol.,* 10**,** 51-57.

MORLEY, S. M., GAYLARDE, P. M. & SARKANY, I. 1985b. Epidermal thickness in systemic sclerosis and morphoea. *Clin Exp Dermatol,* 10**,** 51-7.

MUNGER, K., PIETENPOL, J. A., PITTELKOW, M. R., HOLT, J. T. & MOSES, H. L. 1992. Transforming growth factor beta 1 regulation of c-myc expression, pRB phosphorylation, and cell cycle progression in keratinocytes. *Cell Growth Differ,* 3**,** 291-8.

MUNZ, B., SMOLA, H., ENGELHARDT, F., BLEUEL, K., BRAUCHLE, M., LEIN, I., EVANS, L. W., HUYLEBROECK, D., BALLING, R. & WERNER, S. 1999. Overexpression of activin A in the skin of transgenic mice reveals new activities of activin in epidermal morphogenesis, dermal fibrosis and wound repair. *EMBO J,* 18**,** 5205-15.

MURPHY, C. S., PIETENPOL, J. A., MUNGER, K., HOWLEY, P. M. & MOSES, H. L. 1991. c-myc and pRB: role in TGF-beta 1 inhibition of keratinocyte proliferation. *Cold Spring Harb Symp Quant Biol,* 56**,** 129-35.

MUSTOE, T. A. & GURJALA, A. 2011. The role of the epidermis and the mechanism of action of occlusive dressings in scarring. *Wound Repair Regen,* 19 Suppl 1**,** s16-21.

MUTIS, T., DE BUEGER, M., BAKKER, A. & OTTENHOFF, T. H. 1993. HLA class II+ human keratinocytes present Mycobacterium leprae antigens to CD4+ Th1-like cells. *Scand J Immunol,* 37**,** 43-51.

NACKEN, W., ROTH, J., SORG, C. & KERKHOFF, C. 2003. S100A9/S100A8: Myeloid representatives of the S100 protein family as prominent players in innate immunity. *Microsc Res Tech,* 60**,** 569-80.

NAKAMURA, K., WILLIAMS, I. R. & KUPPER, T. S. 1995. Keratinocyte-derived monocyte chemoattractant protein 1 (MCP-1): analysis in a transgenic model demonstrates MCP-1 can recruit dendritic and Langerhans cells to skin. *J.Invest Dermatol.,* 105**,** 635-643.

NAKAMURA, M. & TOKURA, Y. 2011. Expression of SNAI1 and TWIST1 in the eccrine glands of patients with systemic sclerosis: possible involvement of epithelial-mesenchymal transition in the pathogenesis. *Br.J.Dermatol.,* 164**,** 204-205.

NAYERI, F., XU, J., ABDIU, A., NAYERI, T., AILI, D., LIEDBERG, B. & CARLSSON, U. 2006. Autocrine production of biologically active hepatocyte growth factor (HGF) by injured human skin. *J Dermatol Sci,* 43**,** 49-56.

NEEDLEMAN, B. W., WIGLEY, F. M. & STAIR, R. W. 1992. Interleukin-1, interleukin-2, interleukin-4, interleukin-6, tumor necrosis factor alpha, and interferon-gamma levels in sera from patients with scleroderma. *Arthritis Rheum,* 35**,** 67-72.

NEWTON, R. A. & HOGG, N. 1998. The human S100 protein MRP-14 is a novel activator of the beta 2 integrin Mac-1 on neutrophils. *J Immunol,* 160**,** 1427-35.

NICKOLOFF, B. J. & NAIDU, Y. 1994. Perturbation of epidermal barrier function correlates with initiation of cytokine cascade in human skin. *J Am Acad Dermatol,* 30**,** 535-46.

NIEMANN, C. & WATT, F. M. 2002. Designer skin: lineage commitment in postnatal epidermis. *Trends in Cell Biology,* 12**,** 185-192.

NIESSEN, F. B., ANDRIESSEN, M. P., SCHALKWIJK, J., VISSER, L. & TIMENS, W. 2001. Keratinocyte-derived growth factors play a role in the formation of hypertrophic scars. *J Pathol,* 194**,** 207-16.

NIETERT, P. J. & SILVER, R. M. 2000. Systemic sclerosis: environmental and occupational risk factors. *Curr Opin Rheumatol,* 12**,** 520-6.

NIETERT, P. J., SUTHERLAND, S. E., SILVER, R. M., PANDEY, J. P. & DOSEMECI, M. 1999. Solvent oriented hobbies and the risk of systemic sclerosis. *J Rheumatol,* 26**,** 2369-72.

NIKITOROWICZ-BUNIAK, J., SHIWEN, X., DENTON, C. P., ABRAHAM, D. & STRATTON, R. 2014. Abnormally Differentiating Keratinocytes in the Epidermis of Systemic Sclerosis Patients Show Enhanced Secretion of CCN2 and S100A9. *J Invest Dermatol*.

NODA, S., ASANO, Y., TAKAHASHI, T., AKAMATA, K., AOZASA, N., TANIGUCHI, T., ICHIMURA, Y., TOYAMA, T., SUMIDA, H., KUWANO, Y., YANABA, K., TADA, Y., SUGAYA, M., KADONO, T. & SATO, S. 2013. Decreased cathepsin V expression due to Fli1 deficiency contributes to the development of dermal fibrosis and proliferative vasculopathy in systemic sclerosis. *Rheumatology (Oxford),* 52**,** 790-9.

NOWINSKI, D., LYSHEDEN, A. S., GARDNER, H., RUBIN, K., GERDIN, B. & IVARSSON, M. 2004. Analysis of gene expression in fibroblasts in response to keratinocyte-derived factors in vitro: potential implications for the wound healing process. *J.Invest Dermatol.,* 122**,** 216-221.

NUKUI, T., EHAMA, R., SAKAGUCHI, M., SONEGAWA, H., KATAGIRI, C., HIBINO, T. & HUH, N. H. 2008. S100A8/A9, a key mediator for positive feedback growth stimulation of normal human keratinocytes. *J Cell Biochem,* 104**,** 453-64.

O'KANE, S. & FERGUSON, M. W. 1997. Transforming growth factor beta s and wound healing. *Int J Biochem Cell Biol,* 29**,** 63-78.

ODINK, K., CERLETTI, N., BRUGGEN, J., CLERC, R. G., TARCSAY, L., ZWADLO, G., GERHARDS, G., SCHLEGEL, R. & SORG, C. 1987. Two calcium-binding proteins in infiltrate macrophages of rheumatoid arthritis. *Nature,* 330**,** 80-2.

OHTSUKA, T. & YAMAZAKI, S. 2004. Increased prevalence of human parvovirus B19 DNA in systemic sclerosis skin. *Br J Dermatol,* 150**,** 1091-5.

ONG, C. T., KHOO, Y. T., TAN, E. K., MUKHOPADHYAY, A., DO, D. V., HAN, H. C., LIM, I. J. & PHAN, T. T. 2007. Epithelial–mesenchymal interactions in keloid pathogenesis modulate vascular endothelial growth factor expression and secretion. *The Journal of Pathology,* 211**,** 95-108.

OVERALL, C. M., WRANA, J. L. & SODEK, J. 1989. Independent regulation of collagenase, 72-kDa progelatinase, and metalloendoproteinase inhibitor expression in human fibroblasts by transforming growth factor-beta. *J Biol Chem,* 264**,** 1860-9.

PABLOS, J. L., SANTIAGO, B., GALINDO, M., CARREIRA, P. E., BALLESTIN, C. & GOMEZ-REINO, J. J. 1999. Keratinocyte apoptosis and p53 expression in cutaneous lupus and dermatomyositis. *J.Pathol.,* 188**,** 63-68.

PAGLIA, L., WILCZEK, J., DE LEON, L. D., MARTIN, G. R., HORLEIN, D. & MULLER, P. 1979. Inhibition of procollagen cell-free synthesis by amino-terminal extension peptides. *Biochemistry,* 18**,** 5030-4.

PAN, L. H., YAMAUCHI, K., UZUKI, M., NAKANISHI, T., TAKIGAWA, M., INOUE, H. & SAWAI, T. 2001. Type II alveolar epithelial cells and interstitial fibroblasts express connective tissue growth factor in IPF. *Eur Respir J,* 17**,** 1220-7.

PASPARAKIS, M., SCHMIDT-SUPPRIAN, M. & RAJEWSKY, K. 2002. IkappaB kinase signaling is essential for maintenance of mature B cells. *J Exp Med,* 196**,** 743-52.

PASTORE, S., FANALES-BELASIO, E., ALBANESI, C., CHINNI, L. M., GIANNETTI, A. & GIROLOMONI, G. 1997. Granulocyte macrophage colony-stimulating factor is overproduced by keratinocytes in atopic dermatitis. Implications for sustained dendritic cell activation in the skin. *J Clin Invest,* 99**,** 3009-17.

PERBAL, B. 2004. CCN proteins: multifunctional signalling regulators. *Lancet,* 363**,** 62-4.

PHAN, T. T., LIM, I. J., AALAMI, O., LORGET, F., KHOO, A., TAN, E. K., MUKHOPADHYAY, A. & LONGAKER, M. T. 2005. Smad3 signalling plays an important role in keloid pathogenesis via epithelial–mesenchymal interactions. *The Journal of Pathology,* 207**,** 232-242.

PILI, R., HAGGMAN, M., STADLER, W. M., GINGRICH, J. R., ASSIKIS, V. J., BJORK, A., NORDLE, O., FORSBERG, G., CARDUCCI, M. A. & ARMSTRONG, A. J. 2011. Phase II randomized, double-blind, placebo-controlled study of tasquinimod in men with minimally symptomatic metastatic castrate-resistant prostate cancer. *J Clin Oncol,* 29**,** 4022-8.

PIVARCSI, A., BODAI, L., RETHI, B., KENDERESSY-SZABO, A., KORECK, A., SZELL, M., BEER, Z., BATA-CSORGOO, Z., MAGOCSI, M., RAJNAVOLGYI, E., DOBOZY, A. & KEMENY, L. 2003. Expression and function of Toll-like receptors 2 and 4 in human keratinocytes. *Int Immunol,* 15**,** 721-30.

POORMOGHIM, H., LUCAS, M., FERTIG, N. & MEDSGER, T. A., JR. 2000. Systemic sclerosis sine scleroderma: demographic, clinical, and serologic features and survival in forty-eight patients. *Arthritis Rheum,* 43**,** 444-51.

POPE, J. E., SHUM, D. T., GOTTSCHALK, R., STEVENS, A. & MCMANUS, R. 1996. Increased pigmentation in scleroderma. *J.Rheumatol.,* 23**,** 1912-1916.

POSTIGLIONE, L., LADOGANA, P., MONTAGNANI, S., DI SPIGNA, G., CASTALDO, C., TURANO, M., BRUNO, E. M., DI MEGLIO, F., RICCIO, A. & ROSSI, G. 2005. Effect of granulocyte macrophage-colony stimulating factor on extracellular matrix deposition by dermal fibroblasts from patients with scleroderma. *J Rheumatol,* 32**,** 656-64.

POSTIGLIONE, L., MONTAGNANI, S., RICCIO, A., MONTUORI, N., SCIORIO, S., LADOGANA, P., SPIGNA, G. D., CASTALDO, C. & ROSSI, G. 2002. Enhanced expression of the receptor for granulocyte macrophage colony stimulating factor on dermal fibroblasts from scleroderma patients. *J Rheumatol,* 29**,** 94-101.

PULLERITS, R., JONSSON, I. M., KOLLIAS, G. & TARKOWSKI, A. 2008. Induction of arthritis by high mobility group box chromosomal protein 1 is independent of tumour necrosis factor signalling. *Arthritis Res Ther,* 10**,** R72.

QUERFELD, C., ECKES, B., HUERKAMP, C., KRIEG, T. & SOLLBERG, S. 1999. Expression of TGF-beta 1, -beta 2 and -beta 3 in localized and systemic scleroderma. *J Dermatol Sci,* 21**,** 13-22.

RADIC, M., KALITERNA, D. M., BONACIN, D., VERGLES, J. M., RADIC, J., FABIJANIC, D. & KOVACIC, V. 2012. Is Helicobacter pylori infection a risk factor for disease severity in systemic sclerosis? *Rheumatol Int*.

RADSTAKE, T. R., GORLOVA, O., RUEDA, B., MARTIN, J. E., ALIZADEH, B. Z., PALOMINO-MORALES, R., COENEN, M. J., VONK, M. C., VOSKUYL, A. E., SCHUERWEGH, A. J., BROEN, J. C., VAN RIEL, P. L., VAN 'T SLOT, R., ITALIAANDER, A., OPHOFF, R. A., RIEMEKASTEN, G., HUNZELMANN, N., SIMEON, C. P., ORTEGO-CENTENO, N., GONZALEZ-GAY, M. A., GONZALEZ-ESCRIBANO, M. F., AIRO, P., VAN LAAR, J., HERRICK, A., WORTHINGTON, J., HESSELSTRAND, R., SMITH, V., DE KEYSER, F., HOUSSIAU, F., CHEE, M. M., MADHOK, R., SHIELS, P., WESTHOVENS, R., KREUTER, A., KIENER, H., DE BAERE, E., WITTE, T., PADYKOV, L., KLARESKOG, L., BERETTA, L., SCORZA, R., LIE, B. A., HOFFMANN-VOLD, A. M., CARREIRA, P., VARGA, J., HINCHCLIFF, M., GREGERSEN, P. K., LEE, A. T., YING, J., HAN, Y., WENG, S. F., AMOS, C. I., WIGLEY, F. M., HUMMERS, L., NELSON, J. L., AGARWAL, S. K., ASSASSI, S., GOURH, P., TAN, F. K., KOELEMAN, B. P., ARNETT, F. C., MARTIN, J. & MAYES, M. D. 2010. Genome-wide association study of systemic sclerosis identifies CD247 as a new susceptibility locus. *Nat Genet,* 42**,** 426-9.

RAMMES, A., ROTH, J., GOEBELER, M., KLEMPT, M., HARTMANN, M. & SORG, C. 1997. Myeloid-related protein (MRP) 8 and MRP14, calcium-binding proteins of the S100 family, are secreted by activated monocytes via a novel, tubulin-dependent pathway. *J Biol Chem,* 272**,** 9496-502.

RASANEN, K. & VAHERI, A. 2010. TGF-beta1 causes epithelial-mesenchymal transition in HaCaT derivatives, but induces expression of COX-2 and migration only in benign, not in malignant keratinocytes. *J Dermatol Sci,* 58**,** 97-104.

REISS, M. J., HAN, Y. P. & GARNER, W. L. 2009. Alpha1-antichymotrypsin activity correlates with and may modulate matrix metalloproteinase-9 in human acute wounds. *Wound Repair Regen,* 17**,** 418-26.

RHEINWALD, J. G. & GREEN, H. 1975. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell,* 6**,** 331-43.

RHODES, L. E., HASHIM, I. A., MCLAUGHLIN, P. J. & FRIEDMANN, P. S. 1999. Blister fluid cytokines in cutaneous inflammatory bullous disorders. *Acta Derm Venereol,* 79**,** 288-90.

RIFKIN, D. B. 2005. Latent transforming growth factor-beta (TGF-beta) binding proteins: orchestrators of TGF-beta availability. *J Biol Chem,* 280**,** 7409-12.

ROBERTS, A. B. 1999. TGF-beta signaling from receptors to the nucleus. *Microbes Infect,* 1**,** 1265-73.

ROBITAILLE, H., SIMARD-BISSON, C., LAROUCHE, D., TANGUAY, R. M., BLOUIN, R. & GERMAIN, L. 2010. The small heat-shock protein Hsp27 undergoes ERK-dependent phosphorylation and redistribution to the cytoskeleton in response to dual leucine zipper-bearing kinase expression. *J Invest Dermatol,* 130**,** 74-85.

RODNAN, G. P., LIPINSKI, E. & LUKSICK, J. 1979. Skin thickness and collagen content in progressive systemic sclerosis and localized scleroderma. *Arthritis Rheum,* 22**,** 130-40.

ROGALLA, T., EHRNSPERGER, M., PREVILLE, X., KOTLYAROV, A., LUTSCH, G., DUCASSE, C., PAUL, C., WIESKE, M., ARRIGO, A. P., BUCHNER, J. & GAESTEL, M. 1999. Regulation of Hsp27 oligomerization, chaperone function, and protective activity against oxidative stress/tumor necrosis factor alpha by phosphorylation. *J Biol Chem,* 274**,** 18947-56.

ROSSI, A., SOZIO, F., SESTINI, P., RENZONI, E. A., KHAN, K., DENTON, C. P., ABRAHAM, D. J. & WEBER, E. 2010. Lymphatic and blood vessels in scleroderma skin, a morphometric analysis. *Hum Pathol,* 41**,** 366-74.

ROTH, J., VOGL, T., SORG, C. & SUNDERKOTTER, C. 2003. Phagocyte-specific S100 proteins: a novel group of proinflammatory molecules. *Trends Immunol,* 24**,** 155-8.

RUBIN, J. S., BOTTARO, D. P., CHEDID, M., MIKI, T., RON, D., CUNHA, G. R. & FINCH, P. W. 1995. Keratinocyte growth factor as a cytokine that mediates mesenchymal-epithelial interaction. *EXS,* 74**,** 191-214.

RUDNICKA, L., VARGA, J., CHRISTIANO, A. M., IOZZO, R. V., JIMENEZ, S. A. & UITTO, J. 1994. Elevated expression of type VII collagen in the skin of patients with systemic sclerosis. Regulation by transforming growth factor-beta. *J Clin Invest,* 93**,** 1709-15.

RUEDA, B., BROEN, J., SIMEON, C., HESSELSTRAND, R., DIAZ, B., SUAREZ, H., ORTEGO-CENTENO, N., RIEMEKASTEN, G., FONOLLOSA, V., VONK, M. C., VAN DEN HOOGEN, F. H., SANCHEZ-ROMAN, J., AGUIRRE-ZAMORANO, M. A., GARCIA-PORTALES, R., PROS, A., CAMPS, M. T., GONZALEZ-GAY, M. A., COENEN, M. J., AIRO, P., BERETTA, L., SCORZA, R., VAN LAAR, J., GONZALEZ-ESCRIBANO, M. F., NELSON, J. L., RADSTAKE, T. R. & MARTIN, J. 2009. The STAT4 gene influences the genetic predisposition to systemic sclerosis phenotype. *Hum Mol Genet,* 18**,** 2071-7.

RUEDA, B., GOURH, P., BROEN, J., AGARWAL, S. K., SIMEON, C., ORTEGO-CENTENO, N., VONK, M. C., COENEN, M., RIEMEKASTEN, G., HUNZELMANN, N., HESSELSTRAND, R., TAN, F. K., REVEILLE, J. D., ASSASSI, S., GARCIA-HERNANDEZ, F. J., CARREIRA, P., CAMPS, M., FERNANDEZ-NEBRO, A., GARCIA DE LA PENA, P., NEARNEY, T., HILDA, D., GONZALEZ-GAY, M. A., AIRO, P., BERETTA, L., SCORZA, R., RADSTAKE, T. R., MAYES, M. D., ARNETT, F. C. & MARTIN, J. 2010. BANK1 functional variants are associated with susceptibility to diffuse systemic sclerosis in Caucasians. *Ann Rheum Dis,* 69**,** 700-5.

SARRIO, D., RODRIGUEZ-PINILLA, S. M., HARDISSON, D., CANO, A., MORENO-BUENO, G. & PALACIOS, J. 2008. Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype. *Cancer Res,* 68**,** 989-97.

SATO, S., HASEGAWA, M. & TAKEHARA, K. 2001. Serum levels of interleukin-6 and interleukin-10 correlate with total skin thickness score in patients with systemic sclerosis. *J Dermatol Sci,* 27**,** 140-6.

SATO, S., NAGAOKA, T., HASEGAWA, M., TAMATANI, T., NAKANISHI, T., TAKIGAWA, M. & TAKEHARA, K. 2000. Serum levels of connective tissue growth factor are elevated in patients with systemic sclerosis: association with extent of skin sclerosis and severity of pulmonary fibrosis. *J Rheumatol,* 27**,** 149-54.

SATO, Y. & RIFKIN, D. B. 1989. Inhibition of endothelial cell movement by pericytes and smooth muscle cells: activation of a latent transforming growth factor-beta 1-like molecule by plasmin during co-culture. *J Cell Biol,* 109**,** 309-15.

SAUERMANN, K., GAMBICHLER, T., JASPERS, S., RADENHAUSEN, M., RAPP, S., REICH, S., ALTMEYER, P., CLEMANN, S., TEICHMANN, S., ENNEN, J. & HOFFMANN, K. 2002. Histometric data obtained by in vivo confocal laser scanning microscopy in patients with systemic sclerosis. *BMC Dermatol,* 2**,** 8.

SAVAGNER, P., KUSEWITT, D. F., CARVER, E. A., MAGNINO, F., CHOI, C., GRIDLEY, T. & HUDSON, L. G. 2005. Developmental transcription factor slug is required for effective re-epithelialization by adult keratinocytes. *J Cell Physiol,* 202**,** 858-66.

SCHLINGEMANN, J., HESS, J., WROBEL, G., BREITENBACH, U., GEBHARDT, C., STEINLEIN, P., KRAMER, H., FURSTENBERGER, G., HAHN, M., ANGEL, P. & LICHTER, P. 2003. Profile of gene expression induced by the tumour promotor TPA in murine epithelial cells. *Int J Cancer,* 104**,** 699-708.

SCHMID, P., ITIN, P., CHERRY, G., BI, C. & COX, D. A. 1998. Enhanced expression of transforming growth factor-beta type I and type II receptors in wound granulation tissue and hypertrophic scar. *Am J Pathol,* 152**,** 485-93.

SCHULTZ-CHERRY, S. & MURPHY-ULLRICH, J. E. 1993. Thrombospondin causes activation of latent transforming growth factor-beta secreted by endothelial cells by a novel mechanism. *J Cell Biol,* 122**,** 923-32.

SCHULZE-TOPPHOFF, U., SHETTY, A., VARRIN-DOYER, M., MOLNARFI, N., SAGAN, S. A., SOBEL, R. A., NELSON, P. A. & ZAMVIL, S. S. 2012. Laquinimod, a quinoline-3-carboxamide, induces type II myeloid cells that modulate central nervous system autoimmunity. *PLoS One,* 7**,** e33797.

SHAND, L., LUNT, M., NIHTYANOVA, S., HOSEINI, M., SILMAN, A., BLACK, C. M. & DENTON, C. P. 2007. Relationship between change in skin score and disease outcome in diffuse cutaneous systemic sclerosis: application of a latent linear trajectory model. *Arthritis Rheum,* 56**,** 2422-31.

SHANNON, J. M. & HYATT, B. A. 2004. Epithelial-mesenchymal interactions in the developing lung. *Annu Rev Physiol,* 66**,** 625-45.

SHAPIRA, Y., AGMON-LEVIN, N., SELMI, C., PETRIKOVA, J., BARZILAI, O., RAM, M., BIZZARO, N., VALENTINI, G., MATUCCI-CERINIC, M., ANAYA, J. M., KATZ, B. S.

& SHOENFELD, Y. 2012. Prevalence of anti-Toxoplasma antibodies in patients with autoimmune diseases. *J Autoimmun,* 39**,** 112-6.

SHEPHARD, P., HINZ, B., SMOLA-HESS, S., MEISTER, J. J., KRIEG, T. & SMOLA, H. 2004a. Dissecting the roles of endothelin, TGF-beta and GM-CSF on myofibroblast differentiation by keratinocytes. *Thromb Haemost,* 92**,** 262-74.

SHEPHARD, P., MARTIN, G., SMOLA-HESS, S., BRUNNER, G., KRIEG, T. & SMOLA, H. 2004b. Myofibroblast differentiation is induced in keratinocyte-fibroblast co-cultures and is antagonistically regulated by endogenous transforming growth factor-beta and interleukin-1. *Am J Pathol,* 164**,** 2055-66.

SHERRIFF-TADANO, R., OHTA, A., MORITO, F., MITAMURA, M., HARUTA, Y., KOARADA, S., TADA, Y., NAGASAWA, K. & OZAKI, I. 2006. Antifibrotic effects of hepatocyte growth factor on scleroderma fibroblasts and analysis of its mechanism. *Mod Rheumatol,* 16**,** 364-71.

SHI-WEN, X., KENNEDY, L., RENZONI, E. A., BOU-GHARIOS, G., DU BOIS, R. M., BLACK, C. M., DENTON, C. P., ABRAHAM, D. J. & LEASK, A. 2007. Endothelin is a downstream mediator of profibrotic responses to transforming growth factor beta in human lung fibroblasts. *Arthritis Rheum,* 56**,** 4189-94.

SHI-WEN, X., LEASK, A. & ABRAHAM, D. 2008. Regulation and function of connective tissue growth factor/CCN2 in tissue repair, scarring and fibrosis. *Cytokine Growth Factor Rev,* 19**,** 133-44.

SHI-WEN, X., PENNINGTON, D., HOLMES, A., LEASK, A., BRADHAM, D., BEAUCHAMP, J. R., FONSECA, C., DU BOIS, R. M., MARTIN, G. R., BLACK, C. M. & ABRAHAM, D. J. 2000. Autocrine overexpression of CTGF maintains fibrosis: RDA analysis of fibrosis genes in systemic sclerosis. *Exp Cell Res,* 259**,** 213-24.

SHIBATA, F., MIYAMA, K., SHINODA, F., MIZUMOTO, J., TAKANO, K. & NAKAGAWA, H. 2004. Fibroblast growth-stimulating activity of S100A9 (MRP-14). *Eur J Biochem,* 271**,** 2137-43.

SILMAN, A., JANNINI, S., SYMMONS, D. & BACON, P. 1988. An epidemiological study of scleroderma in the West Midlands. *Br J Rheumatol,* 27**,** 286-90.

SILMAN, A. J. & JONES, S. 1992. What is the contribution of occupational environmental factors to the occurrence of scleroderma in men? *Ann Rheum Dis,* 51**,** 1322-4.

SIMON, F., BERGERON, D., LAROCHELLE, S., LOPEZ-VALLE, C. A., GENEST, H., ARMOUR, A. & MOULIN, V. J. 2011. Enhanced secretion of TIMP-1 by human hypertrophic scar keratinocytes could contribute to fibrosis. *Burns*.

SIMPSON, C. L., PATEL, D. M. & GREEN, K. J. 2011. Deconstructing the skin: cytoarchitectural determinants of epidermal morphogenesis. *Nat Rev Mol Cell Biol,* 12**,** 565-80.

SINGER, A. J. & CLARK, R. A. 1999. Cutaneous wound healing. *N Engl J Med,* 341**,** 738-46.

SMOLA, H., STARK, H. J., THIEKOTTER, G., MIRANCEA, N., KRIEG, T. & FUSENIG, N. E. 1998. Dynamics of basement membrane formation by keratinocyte-fibroblast interactions in organotypic skin culture. *Exp Cell Res,* 239**,** 399-410.

SMOLA, H., THIEKOTTER, G. & FUSENIG, N. E. 1993. Mutual induction of growth factor gene expression by epidermal-dermal cell interaction. *J Cell Biol,* 122**,** 417-29.

SONNYLAL, S., SHI-WEN, X., LEONI, P., NAFF, K., VAN PELT, C. S., NAKAMURA, H., LEASK, A., ABRAHAM, D., BOU-GHARIOS, G. & DE CROMBRUGGHE, B. 2010. Selective expression of connective tissue growth factor in fibroblasts in vivo promotes systemic tissue fibrosis. *Arthritis Rheum,* 62**,** 1523-32.

SONNYLAL, S., XU, S., JONES, H., TAM, A., SREERAM, V. R., PONTICOS, M., NORMAN, J., AGRAWAL, P., ABRAHAM, D. & DE CROMBRUGGHE, B. 2013. Connective tissue growth factor causes EMT-like cell fate changes in vivo and in vitro. *J Cell Sci,* 126**,** 2164-75.

SROUSSI, H. Y., BERLINE, J. & PALEFSKY, J. M. 2007. Oxidation of methionine 63 and 83 regulates the effect of S100A9 on the migration of neutrophils in vitro. *J Leukoc Biol,* 81**,** 818-24.

STEEN, V. D. & MEDSGER, T. A., JR. 2001. Improvement in skin thickening in systemic sclerosis associated with improved survival. *Arthritis Rheum,* 44**,** 2828-35.

STEEN, V. D., MEDSGER, T. A., JR., OSIAL, T. A., JR., ZIEGLER, G. L., SHAPIRO, A. P. & RODNAN, G. P. 1984. Factors predicting development of renal involvement in progressive systemic sclerosis. *Am J Med,* 76**,** 779-86.

STEINHOFF, M., BRZOSKA, T. & LUGER, T. A. 2001. Keratinocytes in epidermal immune responses. *Curr Opin Allergy Clin Immunol,* 1**,** 469-76.

STINGL, G., TAMAKI, K. & KATZ, S. I. 1980. Origin and function of epidermal Langerhans cells. *Immunol Rev,* 53**,** 149-74.

STRANGE, P., SKOV, L. & BAADSGAARD, O. 1994. Interferon gamma-treated keratinocytes activate T cells in the presence of superantigens: involvement of major histocompatibility complex class II molecules. *J Invest Dermatol,* 102**,** 150-4.

SUN, T. T. & GREEN, H. 1976. Differentiation of the epidermal keratinocyte in cell culture: formation of the cornified envelope. *Cell,* 9**,** 511-21.

SUNAHORI, K., YAMAMURA, M., YAMANA, J., TAKASUGI, K., KAWASHIMA, M., YAMAMOTO, H., CHAZIN, W. J., NAKATANI, Y., YUI, S. & MAKINO, H. 2006. The S100A8/A9 heterodimer amplifies proinflammatory cytokine production by macrophages via activation of nuclear factor kappa B and p38 mitogen-activated protein kinase in rheumatoid arthritis. *Arthritis Res Ther,* 8**,** R69.

SZABOWSKI, A., MAAS-SZABOWSKI, N., ANDRECHT, S., KOLBUS, A., SCHORPP-KISTNER, M., FUSENIG, N. E. & ANGEL, P. 2000. c-Jun and JunB antagonistically control cytokine-regulated mesenchymal-epidermal interaction in skin. *Cell,* 103**,** 745-755.

SZOLNOKY, G., BATA-CSORGO, Z., KENDERESSY, A. S., KISS, M., PIVARCSI, A., NOVAK, Z., NAGY NEWMAN, K., MICHEL, G., RUZICKA, T., MARODI, L., DOBOZY, A. & KEMENY, L. 2001. A mannose-binding receptor is expressed on human keratinocytes and mediates killing of Candida albicans. *J Invest Dermatol,* 117**,** 205-13.

TABATA, H., HARA, N., OTSUKA, S., YAMAKAGE, A., YAMAZAKI, S. & KOIBUCHI, N. 2000. Correlation between diffuse pigmentation and keratinocyte-derived endothelin-1 in systemic sclerosis. *Int.J.Dermatol.,* 39**,** 899-902.

TAKAGAWA, S., LAKOS, G., MORI, Y., YAMAMOTO, T., NISHIOKA, K. & VARGA, J. 2003. Sustained activation of fibroblast transforming growth factor-beta/Smad signaling in a murine model of scleroderma. *J Invest Dermatol,* 121**,** 41-50.

TAKAHASHI, M., AKAMATSU, H., YAGAMI, A., HASEGAWA, S., OHGO, S., ABE, M., IWATA, Y., ARIMA, M., MIZUTANI, H., NAKATA, S. & MATSUNAGA, K. 2013.

Epithelial-mesenchymal transition of the eccrine glands is involved in skin fibrosis in morphea. *J Dermatol,* 40**,** 720-5.

TAN, F. K., WANG, N., KUWANA, M., CHAKRABORTY, R., BONA, C. A., MILEWICZ, D. M. & ARNETT, F. C. 2001. Association of fibrillin 1 single-nucleotide polymorphism haplotypes with systemic sclerosis in Choctaw and Japanese populations. *Arthritis Rheum,* 44**,** 893-901.

TAO, J., LI, L., TAN, Z., LI, Y., YANG, J., TIAN, F., WANG, L., REN, Y., XU, G., HE, X., SHEN, G. & TU, Y. 2011. Up-regulation of CC chemokine ligand 20 and its receptor CCR6 in the lesional skin of early systemic sclerosis. *Eur J Dermatol,* 21**,** 731-6.

THIERY, J. P. & SLEEMAN, J. P. 2006. Complex networks orchestrate epithelial-mesenchymal transitions. *Nat Rev Mol Cell Biol,* 7**,** 131-42.

THOREY, I. S., ROTH, J., REGENBOGEN, J., HALLE, J. P., BITTNER, M., VOGL, T., KAESLER, S., BUGNON, P., REITMAIER, B., DURKA, S., GRAF, A., WOCKNER, M., RIEGER, N., KONSTANTINOW, A., WOLF, E., GOPPELT, A. & WERNER, S. 2001. The Ca2+-binding proteins S100A8 and S100A9 are encoded by novel injury-regulated genes. *J Biol Chem,* 276**,** 35818-25.

TIGALONOWA, M., BJERKE, J. R., LIVDEN, J. K. & MATRE, R. 1990. The distribution of Fc gamma RI, Fc gamma RII and Fc gamma R III on Langerhans' cells and keratinocytes in normal skin. *Acta Derm Venereol,* 70**,** 385-90.

TOMASEK, J. J., GABBIANI, G., HINZ, B., CHAPONNIER, C. & BROWN, R. A. 2002. Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol,* 3**,** 349-63.

TORRES, J. E. & SANCHEZ, J. L. 1998. Histopathologic differentiation between localized and systemic scleroderma. *Am J Dermatopathol,* 20**,** 242-5.

TROJANOWSKA, M. 2002. Molecular aspects of scleroderma. *Front Biosci,* 7**,** d608-18.

TUSHER, V. G., TIBSHIRANI, R. & CHU, G. 2001. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci U S A,* 98**,** 5116-21.

UCHI, H., TERAO, H., KOGA, T. & FURUE, M. 2000. Cytokines and chemokines in the epidermis. *J Dermatol Sci,* 24 Suppl 1**,** S29-38.

USUI, M. L., UNDERWOOD, R. A., MANSBRIDGE, J. N., MUFFLEY, L. A., CARTER, W. G. & OLERUD, J. E. 2005. Morphological evidence for the role of suprabasal keratinocytes in wound reepithelialization. *Wound Repair Regen,* 13**,** 468-79.

VALLADEAU, J. & SAELAND, S. 2005. Cutaneous dendritic cells. *Semin Immunol,* 17**,** 273-83.

VAN BON, L., COSSU, M., LOOF, A., GOHAR, F., WITTKOWSKI, H., VONK, M., ROTH, J., VAN DEN BERG, W., VAN HEERDE, W., BROEN, J. C. & RADSTAKE, T. R. 2014. Proteomic analysis of plasma identifies the Toll-like receptor agonists S100A8/A9 as a novel possible marker for systemic sclerosis phenotype. *Ann Rheum Dis,* 73**,** 1585-9.

VAN DEN HOOGEN, F., KHANNA, D., FRANSEN, J., JOHNSON, S. R., BARON, M., TYNDALL, A., MATUCCI-CERINIC, M., NADEN, R. P., MEDSGER, T. A., JR., CARREIRA, P. E., RIEMEKASTEN, G., CLEMENTS, P. J., DENTON, C. P., DISTLER, O., ALLANORE, Y., FURST, D. E., GABRIELLI, A., MAYES, M. D., VAN LAAR, J. M., SEIBOLD, J. R., CZIRJAK, L., STEEN, V. D., INANC, M., KOWAL-BIELECKA, O., MULLER-LADNER, U., VALENTINI, G., VEALE, D. J., VONK, M. C., WALKER, U. A., CHUNG, L., COLLIER, D. H., ELLEN CSUKA, M., FESSLER, B. J., GUIDUCCI, S., HERRICK, A., HSU, V. M., JIMENEZ, S., KAHALEH, B., MERKEL, P. A., SIERAKOWSKI, S., SILVER, R. M., SIMMS, R. W., VARGA, J. & POPE, J. E. 2013. 2013 classification criteria for systemic sclerosis: an American college of rheumatology/European league against rheumatism collaborative initiative. *Ann Rheum Dis,* 72**,** 1747-55.

VAN PRAET, J. T., SMITH, V., HASPESLAGH, M., DEGRYSE, N., ELEWAUT, D. & DE KEYSER, F. 2011. Histopathological cutaneous alterations in systemic sclerosis: a clinicopathological study. *Arthritis Res Ther,* 13**,** R35.

VANDESOMPELE, J., DE PRETER, K., PATTYN, F., POPPE, B., VAN ROY, N., DE PAEPE, A. & SPELEMAN, F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol,* 3**,** RESEARCH0034.

VARGA, J. & JIMENEZ, S. A. 1986. Stimulation of normal human fibroblast collagen production and processing by transforming growth factor-beta. *Biochem Biophys Res Commun,* 138**,** 974-80.

VERRECCHIA, F., MAUVIEL, A. & FARGE, D. 2006. Transforming growth factor-beta signaling through the Smad proteins: role in systemic sclerosis. *Autoimmun Rev,* 5**,** 563-9.

VON MERING, C., HUYNEN, M., JAEGGI, D., SCHMIDT, S., BORK, P. & SNEL, B. 2003. STRING: a database of predicted functional associations between proteins. *Nucleic Acids Res,* 31**,** 258-61.

VON MERING, C., JENSEN, L. J., SNEL, B., HOOPER, S. D., KRUPP, M., FOGLIERINI, M., JOUFFRE, N., HUYNEN, M. A. & BORK, P. 2005. STRING: known and predicted protein-protein associations, integrated and transferred across organisms. *Nucleic Acids Res,* 33**,** D433-7.

VOSS, A., BODE, G., SOPALLA, C., BENEDYK, M., VARGA, G., BOHM, M., NACKEN, W. & KERKHOFF, C. 2011. Expression of S100A8/A9 in HaCaT keratinocytes alters the rate of cell proliferation and differentiation. *FEBS Lett,* 585**,** 440-6.

VOZENIN-BROTONS, M. C., MILLIAT, F., SABOURIN, J. C., DE GOUVILLE, A. C., FRANCOIS, A., LASSER, P., MORICE, P., HAIE-MEDER, C., LUSINCHI, A., ANTOUN, S., BOURHIS, J., MATHE, D., GIRINSKY, T. & AIGUEPERSE, J. 2003. Fibrogenic signals in patients with radiation enteritis are associated with increased connective tissue growth factor expression. *Int J Radiat Oncol Biol Phys,* 56**,** 561-72.

WAELTI, E. R., INAEBNIT, S. P., RAST, H. P., HUNZIKER, T., LIMAT, A., BRAATHEN, L. R. & WIESMANN, U. 1992. Co-culture of human keratinocytes on post-mitotic human dermal fibroblast feeder cells: production of large amounts of interleukin 6. *J Invest Dermatol,* 98**,** 805-8.

WALTER, P. & RON, D. 2011. The unfolded protein response: from stress pathway to homeostatic regulation. *Science,* 334**,** 1081-6.

WANG, T., ZHANG, L., SHI, C., SUN, H., WANG, J., LI, R., ZOU, Z., RAN, X. & SU, Y. 2012. TGF-beta-induced miR-21 negatively regulates the antiproliferative activity but has no effect on EMT of TGF-beta in HaCaT cells. *Int J Biochem Cell Biol,* 44**,** 366-76.

WATT, F. M. 2002. Role of integrins in regulating epidermal adhesion, growth and differentiation. *EMBO J,* 21**,** 3919-26.

WATT, F. M. & FUJIWARA, H. 2011. Cell-extracellular matrix interactions in normal and diseased skin. *Cold Spring Harb.Perspect.Biol.,* 3.

WATT, F. M. & GREEN, H. 1981. Involucrin synthesis is correlated with cell size in human epidermal cultures. *J.Cell Biol.,* 90**,** 738-742.

WATT, F. M. & HUCK, W. T. 2013. Role of the extracellular matrix in regulating stem cell fate. *Nat Rev Mol Cell Biol,* 14**,** 467-73.

WATT, F. M., JORDAN, P. W. & O'NEILL, C. H. 1988. Cell shape controls terminal differentiation of human epidermal keratinocytes. *Proc Natl Acad Sci U S A,* 85**,** 5576-80.

WATT, F. M., KUBLER, M. D., HOTCHIN, N. A., NICHOLSON, L. J. & ADAMS, J. C. 1993. Regulation of keratinocyte terminal differentiation by integrin-extracellular matrix interactions. *J Cell Sci,* 106 ( Pt 1)**,** 175-82.

WEI, J., BHATTACHARYYA, S., TOURTELLOTTE, W. G. & VARGA, J. 2011. Fibrosis in systemic sclerosis: emerging concepts and implications for targeted therapy. *Autoimmun Rev,* 10**,** 267-75.

WENG, J., MOHAN, R. R., LI, Q. & WILSON, S. E. 1997. IL-1 upregulates keratinocyte growth factor and hepatocyte growth factor mRNA and protein production by cultured stromal fibroblast cells: interleukin-1 beta expression in the cornea. *Cornea,* 16**,** 465-71.

WERNER, S. & GROSE, R. 2003. Regulation of wound healing by growth factors and cytokines. *Physiol Rev,* 83**,** 835-70.

WERNER, S., KRIEG, T. & SMOLA, H. 2007. Keratinocyte-fibroblast interactions in wound healing. *J Invest Dermatol,* 127**,** 998-1008.

WHITFIELD, M. L., FINLAY, D. R., MURRAY, J. I., TROYANSKAYA, O. G., CHI, J. T., PERGAMENSCHIKOV, A., MCCALMONT, T. H., BROWN, P. O., BOTSTEIN, D. & CONNOLLY, M. K. 2003. Systemic and cell type-specific gene expression patterns in scleroderma skin. *Proc Natl Acad Sci U S A,* 100**,** 12319-24.

WILLIS, B. C., LIEBLER, J. M., LUBY-PHELPS, K., NICHOLSON, A. G., CRANDALL, E. D., DU BOIS, R. M. & BOROK, Z. 2005. Induction of epithelial-mesenchymal transition in alveolar epithelial cells by transforming growth factor-beta1: potential role in idiopathic pulmonary fibrosis. *Am J Pathol,* 166**,** 1321-32.

WIPFF, J., AVOUAC, J., LE CHARPENTIER, M., VARRET, M., HOUTTEMAN, A., RUIZ, B., VACHER-LAVENU, M. C., KAHAN, A., BOILEAU, C. & ALLANORE, Y. 2010. Dermal tissue and cellular expression of fibrillin-1 in diffuse cutaneous systemic sclerosis. *Rheumatology (Oxford),* 49**,** 657-61.

WITTMANN, M., PURWAR, R., HARTMANN, C., GUTZMER, R. & WERFEL, T. 2005. Human keratinocytes respond to interleukin-18: implication for the course of chronic inflammatory skin diseases. *J Invest Dermatol,* 124**,** 1225-33.

XIA, W., PHAN, T. T., LIM, I. J., LONGAKER, M. T. & YANG, G. P. 2004. Complex epithelial-mesenchymal interactions modulate transforming growth factor-beta expression in keloid-derived cells. *Wound Repair Regen,* 12**,** 546-56.

XU, W. D., LEROY, E. C. & SMITH, E. A. 1991. Fibronectin release by systemic sclerosis and normal dermal fibroblasts in response to TGF-beta. *J Rheumatol,* 18**,** 241-6.

XU, X., CHEN, H., ZHU, X., MA, Y., LIU, Q., XUE, Y., CHU, H., WU, W., WANG, J. & ZOU, H. 2013a. S100A9 promotes human lung fibroblast cells activation through RAGE mediated ERK1/2 MAP-kinase and NF-kappaB dependent pathways. *Clin Exp Immunol*.

XU, X., WU, W. Y., TU, W. Z., CHU, H. Y., ZHU, X. X., LIANG, M. R., XUE, Y., WANG, J. C. & ZOU, H. J. 2013b. Increased expression of S100A8 and S100A9 in patients with diffuse cutaneous systemic sclerosis. A correlation with organ involvement and immunological abnormalities. *Clin Rheumatol*.

YAMAKAGE, A., KIKUCHI, K., SMITH, E. A., LEROY, E. C. & TROJANOWSKA, M. 1992. Selective upregulation of platelet-derived growth factor alpha receptors by transforming growth factor beta in scleroderma fibroblasts. *J Exp Med,* 175**,** 1227-34.

YAMAMOTO, T. 2008. Pathogenic role of CCL2/MCP-1 in scleroderma. *Front Biosci,* 13**,** 2686-95.

YAN, C., GRIMM, W. A., GARNER, W. L., QIN, L., TRAVIS, T., TAN, N. & HAN, Y. P. 2010. Epithelial to mesenchymal transition in human skin wound healing is induced by tumor necrosis factor-alpha through bone morphogenic protein-2. *Am.J.Pathol.,* 176**,** 2247-2258.

YANG, L., CHAN, T., DEMARE, J., IWASHINA, T., GHAHARY, A., SCOTT, P. G. & TREDGET, E. E. 2001. Healing of burn wounds in transgenic mice overexpressing transforming growth factor-beta 1 in the epidermis. *Am J Pathol,* 159**,** 2147-57.

YASUKAWA, A., HOSOKI, K., TODA, M., MIYAKE, Y., MATSUSHIMA, Y., MATSUMOTO, T., BOVEDA-RUIZ, D., GIL-BERNABE, P., NAGAO, M., SUGIMOTO, M.,

HIRAGUCHI, Y., TOKUDA, R., NAITO, M., TAKAGI, T., D'ALESSANDRO-GABAZZA, C. N., SUGA, S., KOBAYASHI, T., FUJISAWA, T., TAGUCHI, O. & GABAZZA, E. C. 2013. Eosinophils promote epithelial to mesenchymal transition of bronchial epithelial cells. *PLoS One,* 8**,** e64281.

YAZAWA, N., FUJIMOTO, M., KIKUCHI, K., KUBO, M., IHN, H., SATO, S., TAMAKI, T. & TAMAKI, K. 1998. High seroprevalence of Helicobacter pylori infection in patients with systemic sclerosis: association with esophageal involvement. *J Rheumatol,* 25**,** 650-3.

YU, Q. & STAMENKOVIC, I. 2000. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev,* 14**,** 163-76.

YUI, S., MIKAMI, M., TSURUMAKI, K. & YAMAZAKI, M. 1997. Growth-inhibitory and apoptosis-inducing activities of calprotectin derived from inflammatory exudate cells on normal fibroblasts: regulation by metal ions. *J Leukoc Biol,* 61**,** 50-7.

ZAKRZEWSKA, K., CORCIOLI, F., CARLSEN, K. M., GIUGGIOLI, D., FANCI, R., RINIERI, A., FERRI, C. & AZZI, A. 2009. Human parvovirus B19 (B19V) infection in systemic sclerosis patients. *Intervirology,* 52**,** 279-82.

ZAVADIL, J. & BOTTINGER, E. P. 2005. TGF-beta and epithelial-to-mesenchymal transitions. *Oncogene,* 24**,** 5764-5774.

ZEISBERG, M. & NEILSON, E. G. 2009. Biomarkers for epithelial-mesenchymal transitions. *J.Clin.Invest,* 119**,** 1429-1437.

ZEISBERG, M., YANG, C., MARTINO, M., DUNCAN, M. B., RIEDER, F., TANJORE, H. & KALLURI, R. 2007. Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition. *J Biol Chem,* 282**,** 23337-47.

ZENZ, R., EFERL, R., KENNER, L., FLORIN, L., HUMMERICH, L., MEHIC, D., SCHEUCH, H., ANGEL, P., TSCHACHLER, E. & WAGNER, E. F. 2005. Psoriasis-like skin disease and arthritis caused by inducible epidermal deletion of Jun proteins. *Nature,* 437**,** 369-75.

ZHOU, L., ASKEW, D., WU, C. & GILLIAM, A. C. 2007. Cutaneous gene expression by DNA microarray in murine sclerodermatous graft-versus-host disease, a model for human scleroderma. *J Invest Dermatol,* 127**,** 281-92.

www.invivogen.com/review-damage-associated-molecular-patterns (accessed on 01/11/13)

www.phosphosite.com (accessed 10/02/14 – 12/02/2014)

# Appendixes

**Appendix 1. List of primers used in qPCR assays.**

| Symbol | Accession number | Name | Function | Primer sequence |
|---|---|---|---|---|
| Housekeeping genes | | | | |
| ACTB | NM_001101 | Beta actin | Cytoskeletal structural protein | Forward: CACCATGTACCCTGGCATT<br>Reverse: CCGATCCACACGGAGTA |
| B2M | NM_004048 | Beta-2-microglobulin | Beta-chain of major histocompatibility complex class I molecules | Forward: CTCTCTCTTTCTGGCCTGGAG<br>Reverse: ACCCAGACACATAGCAATTCAG |
| SDHA | NM_004168 | Succinate dehydrogenase complex, subunit A | Electron transporter in the TCA cycle and respiratory chain | Forward: AGAAGCCCTTTGAGGAGCA<br>Reverse: CGATCACGGGTCTATATTCCAGA |
| TUBB | NM_178014 | Tubulin | Major constituent of microtubules | Forward: ACATACCTTGAGGCGAGCAA<br>Reverse: TCACTGATCACCTCCCAGAA |
| TBP | NM_003194 | TATA box binding protein | General RNA polymerase II transcription factor | Forward: GAACATCATGGATCAGAACAACAG<br>Reverse: ATAGGGATTCCGGGAGTCAT |
| Genes of interest | | | | |
| COL1A1 | NM_000088.3 | Collagen type I alpha 1 | Extracellular matrix protein | Forward: CCCCTGGAAAGAATGGAGAT<br>Reverse: AATCCTCGAGCACCCTGA |
| CTGF/CTGF | NM_001901.2 | Connective tissue growth factor | Pro-fibrotic | Forward: GACCTGGAAGAGAACATTAAGAAGG<br>Reverse: TCGGTATGTCTTCATGCTGGTG |

| | | | | |
|---|---|---|---|---|
| IL6 | NM_000600.3 | Interleukin 6 | Pro-inflammatory | Forward:<br>GGTACATCCTCGACGGCATCT<br><br>Reverse:<br>GTGCCTCTTTGCTGCTTTCAC |
| IL8 | NM_000584.3 | Interleukin 8 | Pro-inflammatory | Forward:<br>CAGAGACAGCAGAGCACACA<br><br>Reverse:<br>GGCAAAACTGCACCTTCACA |
| SNAI1 | NM_005985 | Snail homolog 1 | Transcriptional repressor of epithelial genes | Forward:<br>CAGGACTCTAATCCAGAGTTTAC CT<br><br>Reverse:<br>ACAGAGTCCCAGATGAGCATTG |
| SNAI2 | NM_003068 | Snail homolog 2 | Transcriptional repressor of E-box motifs incl. E-cadherin | Forward:<br>GAACTGGACACACATACAGTGAT<br><br>Reverse:<br>GGTAGTCCACACAGTGATGG |
| S100A9/ MIF/MRP 14 | NM_002965 | S100 calcium binding protein A9 | Calcium binding; pro-inflammatory DAMP | Forward:<br>TGGCTCCTCGGCTTTGACAGAGT<br><br>Reverse:<br>TGGGTGCCCCAGCTTCACAGA |
| SERPINE 1/ PAI-1 | NM_000602.4 & NM_0011654 13.2 | Serpin peptidase inhibitor/ plasminogen activator inhibitor type 1, | Inhibitor of ECM proteases (uPA/tPA/plasmin/ MMP) | Forward:<br>TCTGAAAAGTCCACTTGCTTGAC<br>Reverse:<br>GCTATGGGATTCAAGATTGATGA CA |
| TLR4 | NM_138554 | Toll-like receptor 4 | Pathogen recognition and activation of immune response | Forward:<br>CTTCTCAACCAAGAACCTGGAC<br><br>Reverse:<br>TTGTCTGGATTTCACACCTGGA |
| NOV | NM_002514.3 | Nephroblastoma overexpressed | The CCN family member important in fibrosis | Forward:<br>TACTGCCTGAGCCTAACTGC<br>Reverse:<br>CCTGTAAGCTGCAAGGGTAA |

## Appendix 2. Protocol for Isolation and Culture of Primary Human Keratinocytes

On the day of biopsy 2 million γ-irradiated 3T3 fibroblasts were seed in T25 flask in the keratinocyte culture media consisting of modified DMEM/F12 (PAA), 10% FBS, RM+ supplement (with final concentration (vol/vol) of 5μg/ml transferrin, 0.4μg/ml hydrocortisone, 1nM cholera toxin, 10ng/ml EGF, 5μg/ml insulin and 0.2nM liothyronine), L-glutamine, 50U/ml penicillin and 50mg/ml streptomycin) and incubated at 10% $CO_2$.

After removing excess fat the skin biopsy sample was transfer into 2.5mg/ml dispase made in PBS and incubated overnight at 4°C. The next day skin was washed in PBS, and transferred into versene where the epidermis was separate form the dermis. The epidermal sheet was then cut, transferred to trypsin/versene and incubated at 37°C. Every 2-3 min, the solution was pipetted up and down to aid keratinocyte detachment. The detached cells were transferred to the keratinocyte culture media, while fresh trypsin/versene was added to the epidermal sheet. The process was continued until complete disintegration of the epidermis. Cells suspension was then centrifuged at 300 x g for 5 min and after re-suspension in the media cells were put in a flask with the pre-seeded γ-irradiated 3T3 and cultured at 5% $CO_2$. Cultures were checked daily, media changed approximately every 3 days, and fresh γ-irradiated 3T3s were added if necessary.

Once large colonies have formed or cells appear to be differentiating feeders were removed with versene, and keratinocytes trypsinised. Cells were then seed cells in flask with γ-irradiated 3T3 for further expansion or cryopreserved.